SYNTHEGO | Lonza

Case Study

Synthetic sgRNAs Enable Researchers to Study Viral Infection in Resting Human CD4+ T Cells

Featuring

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APPLICATION
Bioengineering

RESEARCH AREA
Infectious Disease

PRODUCT
Synthetic sgRNA

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> ***We initially started experimenting with guides from some other companies. However, Synthego's modified single guide RNAs always worked best in our hands, so we decided to move ahead entirely with Synthego. We always use a combination of two guide RNAs for best efficiency.***

At A Glance

Executive Summary

Dr. Manuel Albanese and his colleagues used Synthego's synthetic single guide RNA (sgRNA) and Lonza's 4D-Nucleofector® System to optimize Nucleofection® Conditions in resting human CD4+ T cells. Dr. Albanese conducted this study as a postdoctoral researcher together with Adrian Ruhle in Dr. Oliver Keppler's lab at the Max von Pettenkofer Institute and Gene Center in Munich, Germany. The study was published in *Nature Methods* in January 2022, titled, "Rapid, efficient and activation-neutral gene editing of polyclonal primary human resting CD4+ T cells allows complex functional analyses".

Introduction

Resting CD4+ T cells are highly resistant to human immunodeficiency virus-1 (HIV-1) infection, present multiple blocks to active HIV-1 replication, and often act as reservoirs of latent HIV-1 infection. As such, it is important to study the biology of these cells and mimic the viral interaction and replication steps in naïve, resting CD4+ T cells. The authors targeted multiple genes related to viral infectivity (up to six genes were knocked out simultaneously at rates greater than 98% while maintaining high viability), knocked in large reporter gene segments, and performed downstream functional assays to characterize their various gene edits.

Challenge

Previous attempts at gene editing of resting CD4+ T cells have been met with minimal success and low cell viability. Conventionally, it is challenging to maintain the resting states in CD4+ T cells subsequent to gene knockout, thereby limiting the analysis of pathways and factors that trigger CD4+ T cell activation. This is particularly challenging within the context of HIV-1 infection as it restricts the study of pro- and anti-viral infection factors in host immune cells.

Solution

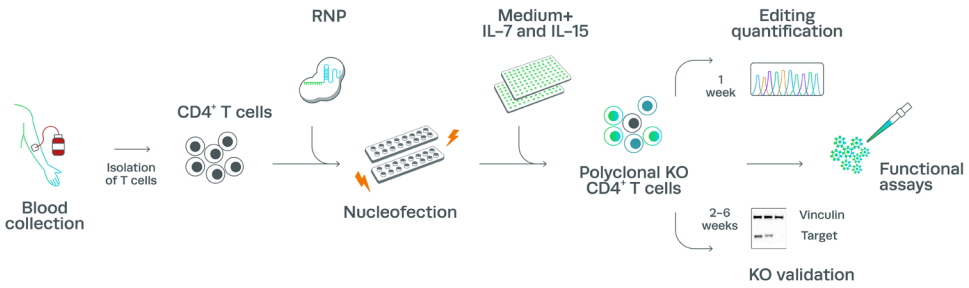
Through a combination of optimized culturing conditions such as interleukin supplementation, Synthego's sgRNA, and Lonza's 4D-Nucleofector® System, the authors were able to optimize the editing conditions for resting human CD4+ T cells and maintain high cell viability for several weeks post Nucleofection®. These edited CD4+ T cells presented a robust system to evaluate the impact of these individual genes on HIV infectivity in resting T cells through a series of downstream functional assays such as T cell migration and fluorescence-activated cell sorting for fluorescence-expressing HIV vectors.

Introduction

Resting human CD4+ T cells are highly resistant to active HIV-1 replication and act as reservoirs of latent infection. As such, it is critically important to study the biology of these cells in naïve, resting CD4+ T cells. The systemic interrogation of the role of canonical genes in viral infection has been challenging as these cells are not compatible with standard transfection protocols and often result in low cell viability following gene editing. Unlike activated CD4+ T cells, resting CD4+ T cells do not proliferate, thus necessitating a highly efficient protocol for generating knockout CD4+ T cells. Previous studies in the field used activated human CD4+ T cells for editing and allowed them to return to resting physiological states for analysis. However, a caveat of using post-activated resting CD4+ T cells is that they do not completely recapitulate the naïve T cell physiology. This has greatly limited scientists from studying the different factors and pathways involved with viral entry and replication in resting CD4+ T cells.

The present case study describes Dr. Albanese's optimized methods to transfect resting human CD4+ T cells using Synthego's single guide RNAs and Lonza's 4D-Nucleofector® System. Using the optimized transfection conditions, Dr. Albanese could knock out up to six genes (CD46, CXCR4, PSGL-1, CD4, TRIM5a, and CPSF6) simultaneously in resting CD4+ T cells with high editing efficiencies and maintained high cellular survivability for several weeks following gene editing. This allowed the authors to interrogate the functionality of these genes in resting CD4+ T cells in a series of downstream assays. Furthermore, the authors utilized this optimized protocol to knock in a green fluorescent protein (GFP) to key genes that regulate viral infection to study alternative pathways for HIV-1 infectivity through simple assays such as fluorescent-activated cell sorting.

Overall, the authors conclude that the impact of this study opens new avenues to investigate virus and human CD4+ T cell interactions and viral latency in physiologically relevant resting CD4+ T cells.



Pipeline Schematic to Establish Polyclonal Knockouts in Human Resting CD4+ T Cells
*The above and following figures were adapted from the original article from Albanese et al, Nat Methods 19, 81–89 (2022) and is licensed under the Creative Commons Attribution License 4.0 which permits the use, sharing, distribution, and reproduction in any format so long as appropriate credit is given to the original authors.

Methods

Dr. Albanese chose Synthego's synthetic sgRNAs and Lonza's 4D Nucleofector® System for his experiments, in combination with optimal cell culture media conditions. This enabled the team to obtain the following:

- Achieve high and consistent knockout efficiencies in resting primary human CD4+ T cells
- Consistent single gene knockout efficiency enabled experiments studying multi-gene knockouts
- High viability in resting human CD4+ T cells following transfection (unlike prior studies that were limited to 72 hours)
- Ability to perform downstream functional studies due to sustained high viability

Synthego's synthetic sgRNA addressed one of the major challenges for Dr. Albanese's study, by providing consistently high knockout efficiencies (>98%) in resting human CD4+ T cells. This was also critical to the downstream functional characterization studies.

Dr. Albanese used a combination of two synthetic sgRNAs for each target gene knockout in order to achieve the best editing efficiency. For delivery into resting CD4+ T cells Dr. Albanese used the 16-well strip of the Lonza 4D-Nucleofector® X Unit. In a total volume of 20 µl, he transfected $2x10^6$ cells with the pre-complexed RNPs. The nuanced pulsing of the 4D-Nucleofector® allowed him to gain high editing efficiencies while maintaining a high viability.

Please refer to the detailed protocol on how to edit resting CD4+ T cells.

Design Your Own Synthetic sgRNA

Synthego's highly pure synthetic sgRNAs ensure high experimental reproducibility. Input your own gRNA sequence or use Synthego's CRISPR Design Tool to start your CRISPR experiment today.

Synthego.com/products/crispr-kits/synthetic-sgrna

Results

Dr. Albanese's work describes a robust approach to editing primary human resting CD4+ T cells through a combination of Synthego's highly efficient synthetic sgRNAs to ensure successful editing, Lonza's 4D-Nucleofector®, and optimal culturing conditions to ensure high cell survival following Nucleofection®. This optimized approach allows for a greater number of available edited cells for the study of both previously evaluated and novel mechanisms of infection in resting CD4+ T cells.

CD4+ T Cells Retain High Viability Following Single and Multi–gene Gene Knockouts

Dr. Albanese and his team demonstrated robust single and multi-gene knockout of various genes integral for viral infection (CD46, CXCR4, PSGL-1, CD4, TRIM5a, and CPSF6) in resting CD4+ T cells (**Figs 1a-b**). Importantly, cell viability remained relatively high following single and multi-gene knockout (50-80% for 4 weeks following infection, **Fig 1c**). Upon activation, edited T cells continued to express canonical activated T cell markers CD25, CD39 CD69, and HLA-DR (**Fig 1d**).

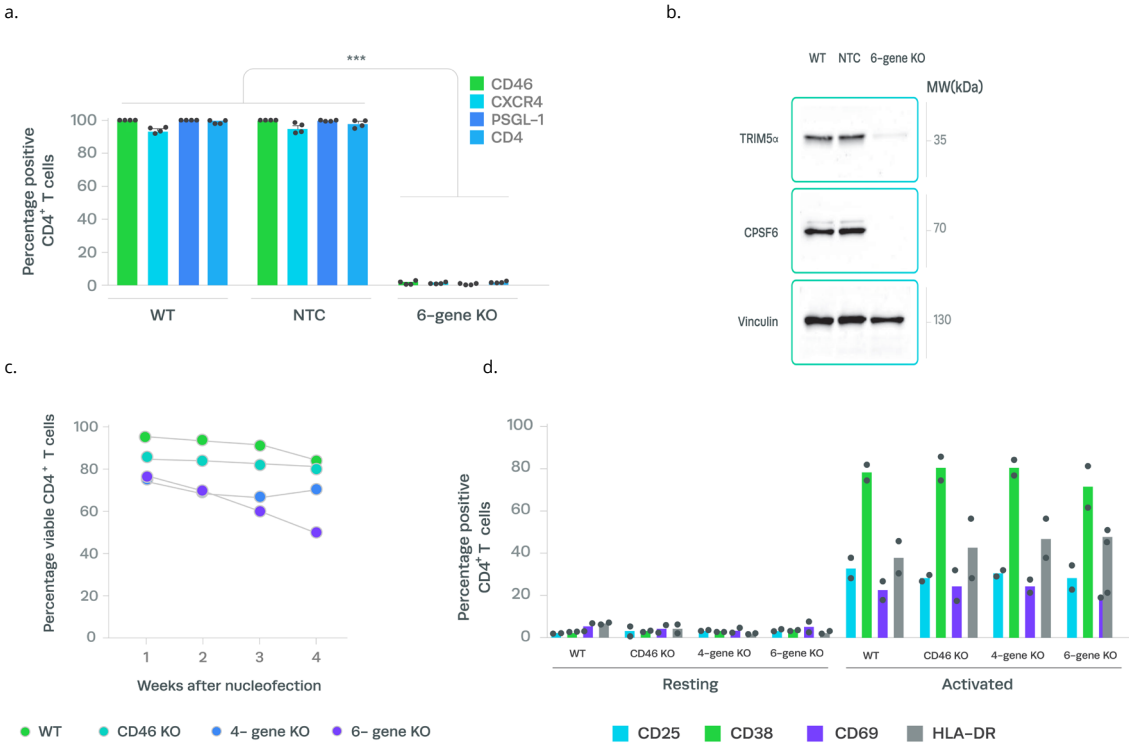


Figure 1. Highly Efficient Multi-gene KO in Primary Human Resting CD4+ T Cells
Simultaneous, polyclonal six-gene knockout following a single RNP nucleofection with Synthego's sgRNAs resulted in the depletion of 4 cell surface markers (CD46, CXCR4, PSGL-1, and CD4) within 2 weeks via fluorescent-activated cell sorting (**a**) and depletion of cytoplasmic proteins (TRIM5a and CPSF6) within 25 days via immunoblotting (**b**). Viability of both single gene and multi-gene (4- and 6-gene) knockout demonstrated high cell survival 4 weeks following nucleofection as compared to wild-type (WT) controls (**c**). Multi-gene-edited CD4+ T cells demonstrated a typical activated CD4+ T cell profile following stimulation (T-Activator CD3/CD28 beads and IL-2 medium) as assessed by canonical active CD4+ T cell markers CD25, CD69, CD38, and HLA-DR via fluorescent activated cell sorting 2 weeks following nucleofection (**d**). (Figure adapted from Albanese et al, Nat Methods 19, 81–89 (2022).

Functional Characterization of Single Gene Knockouts

Single gene KOs were systematically evaluated for their role in HIV-1 infectivity. Cell surface marker CXCR4 is well-known for its role in T cell chemotaxis and HIV-1 entry and infection. The knockout of CXCR4 gene resulted in both a reduction in the ability of CD4+ T cells to migrate and also for its ability to be infected by a GFP-expressing HIV-1 (**Figs 2a-b**).

Dr. Albanese and his team further evaluated transcription factors implicated in HIV-1 infection (MX2, CPSF6, PSGL-1) that had been studied in other cell systems but not resting CD4+ T cells. Their work showed that CPSF6 did not mediate pre- and post-entry of HIV-1 into host CD4+ cells. MX2 and CPSF6 are both noted for their role in interacting with the HIV-1 capsid during nuclear entry of the pre-integration complex and proviral integration. Interestingly, the depletion of CPSF6, but not MX2, resulted in resting CD4+ T cells being refractory toward HIV-1 infection (**Fig 2c**). These are novel findings in the field of resting human CD4+ infectivity by HIV-1.

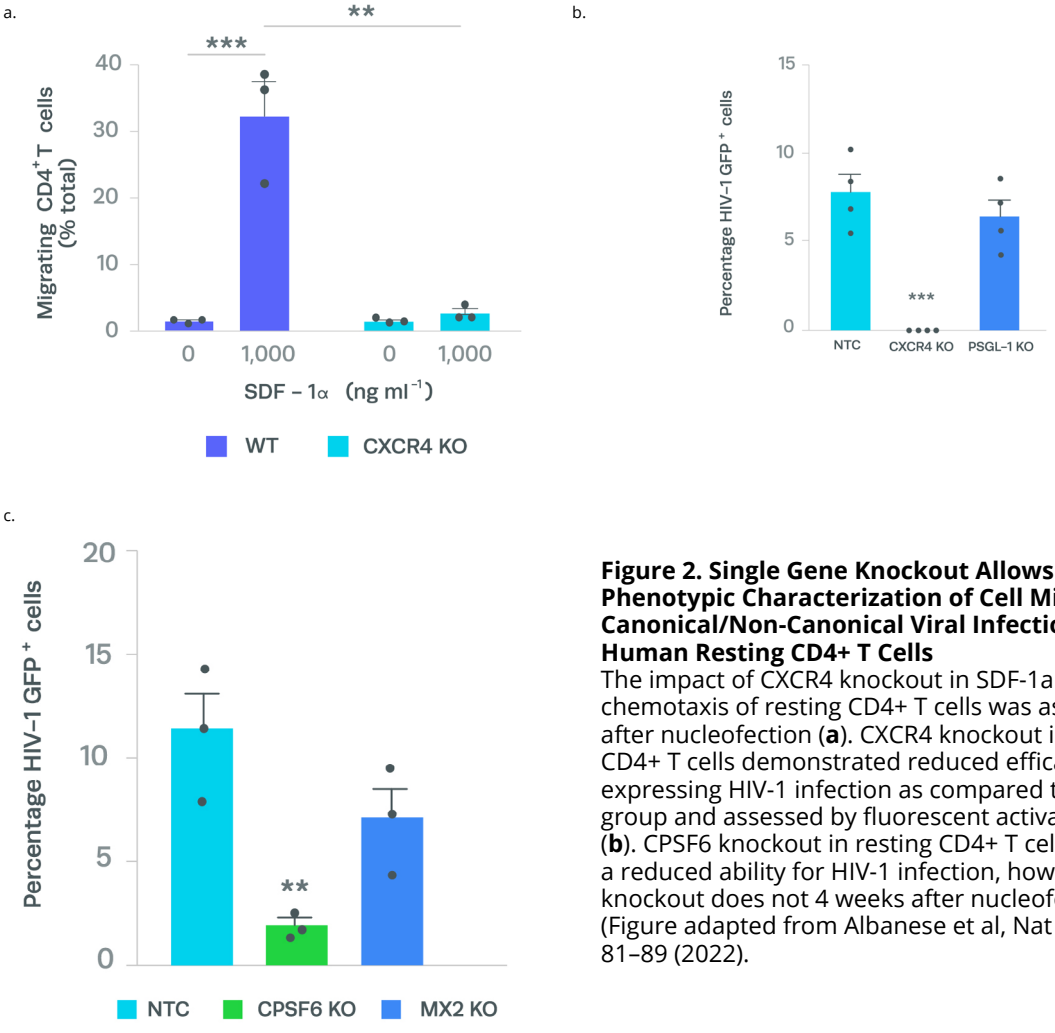


Figure 2. Single Gene Knockout Allows for Phenotypic Characterization of Cell Migration and Canonical/Non-Canonical Viral Infection in Primary Human Resting CD4+ T Cells
The impact of CXCR4 knockout in SDF-1a (CXCL12)-driven chemotaxis of resting CD4+ T cells was assessed 1 week after nucleofection (**a**). CXCR4 knockout in resting CD4+ T cells demonstrated reduced efficacy of GFP-expressing HIV-1 infection as compared to the NTC group and assessed by fluorescent activated cell sorting (**b**). CPSF6 knockout in resting CD4+ T cells demonstrate a reduced ability for HIV-1 infection, however, MX2 knockout does not 4 weeks after nucleofection (**c**). (Figure adapted from Albanese et al, Nat Methods 19, 81–89 (2022).

eGFP Knock–In Upstream of SAMHD1 Enables Studying Alternative Pathways for HIV–1 Infection

Dr. Albanese created a functional reporter system to study the mechanism of SAMHD1 in regulating viral infection. SAMHD1 is a human anti-viral protein that prevents viral replication of HIV-1. The specific knockout of the SAMHD1 gene has been studied in other diseases, and loss-of-function mutations in SAMHD1 have resulted in a greater vulnerability to viral infection.

In order to study the role of SAMHD1 in resting CD4+ T cells, Dr. Albanese and team knocked in an N-terminal eGFP protein into the SAMHD1 gene by using one sgRNA from Synthego and a double-stranded plasmid DNA donor template with approximately 550 base pair homology arms (**Fig 3a**). This resulted in a 2.3% knock-in efficiency of GFP into T cells that were then subsequently enriched for downstream assays via FACS. This GFP-SAMHD1 CD4+ T cell line was then used to study alternative infectivity pathways with high resolution by the researchers. The team used a reporter HIV-1-BFP/SAMHD1-GFP CD4+ T cell system where BFP and GFP were used to demonstrate shifts in the status of T cells following infection.

GFP-SAMHD1 resting CD4+T cells were largely refractory to infection by a BFP-expressing HIV-1 vector unless a known degrader for SAMHD1, Vpx, was introduced which resulted in a decrease in the number of SAMHD1-GFP+ cells and a concomitant increase in HIV-1-BFP+ cells (**Fig 3b, left 4 panels**). To further explore additional pathways of HIV-1 infectivity, Dr. Albanese and colleagues introduced EFV, a reverse transcriptase inhibitor, to Vpx-treated GFP-SAMHD1 CD4+ T cells resulting in an abrogation of BFP+ cells while still maintaining a depletion of GFP+ T cells (**Fig 3b, right 2 panels**) thus allowing for high-resolution analysis of alternative pathways for viral infection.

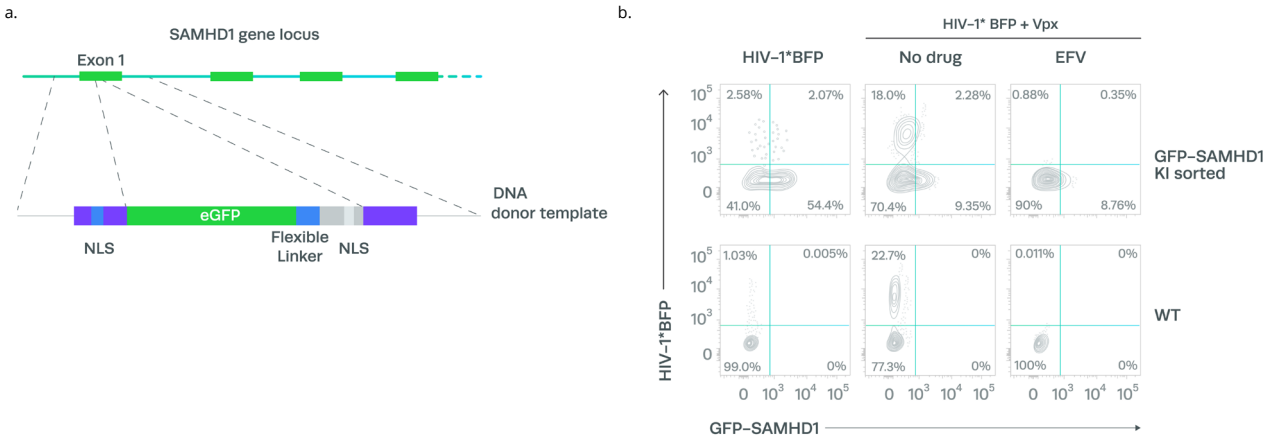


Figure 3. Large Knock-in of eGFP into anti-viral protein SAMHD1 Loci Allows for Study of HIV-1 Infection in Primary Human Resting CD4+ T Cells
SAMHD1, an innate human protein that blocks viral replication of HIV-1 in dendritic cells, macrophages, monocytes, and resting CD4+ lymphocytes, was studied for its role in CD4+ T cell infectivity by HIV-1. eGFP was knocked in upstream of the SAMHD1 loci utilizing a double-stranded plasmid DNA donor template with 550 bp homology arms for exon 1 of SAMHD1 to generate GFP-SAMHD1 expressing resting CD4+ T cells (**a**). Infection of the GFP-SAMHD1 CD4+ T cells with BFP-expressing HIV-1 virions is inhibited except when Vpx protein is introduced which degrades SAMHD1 protein (**b, middle column**). The inclusion of EFV, a reverse transcriptase inhibitor, resulted in an inhibition of BFP-HIV-1 infection despite SAMHD1 depletion (**b, right column**). (Figure adapted from Albanese et al, Nat Methods 19, 81–89 (2022).

Synthego's Synthetic Single Guide RNAs Enable High-Efficiency Knockouts in Resting CD4+ T Cells

When Dr. Albanese and his team compared Synthego's synthetic sgRNAs (freshly prepared or frozen and then thawed) against another major vendor's synthetic sgRNAs, Synthego's sgRNAs resulted in consistently greater (more than 2x) KO efficiency than the other vendor's (**Fig 4**).

This efficient gene editing in resting CD4+ T cells is important as these assays require the use of valuable donor cells from humans. A gene-editing protocol that results in **high cell viability and high gene editing efficiency** is critical in enabling downstream experiments. Furthermore, high editing efficiency is crucial for future downstream clinical applications. A large amount of successfully edited donor cells will impact how many cells are available for patient use. To ensure enough viable cells for the downstream functional assays, Dr. Albanese and the team started out with 2.5x more cells than typically required for these assays.

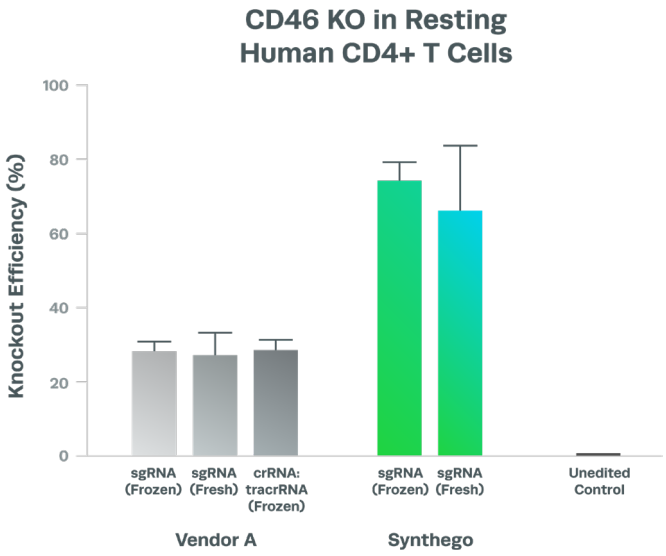


Figure 4. Synthego's Synthetic Guides Outperform Another Vendor's in Editing Resting CD4+ T Cells
The editing efficiency of both frozen and freshly prepared Synthego synthetic sgRNAs were directly compared against another vendor's sgRNA and crRNA:tracrRNA in editing the CD46 loci in resting CD4+ T cells. Synthego's synthetic sgRNAs demonstrated consistently high (60%+) knockout efficiency as assessed by TIDE analysis regardless of preparation condition. (Unpublished data, Albanese et al.)

About Synthego

Synthego is a genome engineering company that enables the acceleration of life science research and development in the pursuit of improved human health. With its foundations in engineering, the company leverages machine learning, automation, and gene editing to build platforms to advance both basic research and therapeutic development programs.

Conclusions

Previous viral interaction studies have utilized CRISPR editing approaches in post-activated CD4+ T cells as they can be readily expanded following gene editing, whereas maintaining viability in resting CD4+ T cells post-gene editing is a persistent problem that has challenged the field. Also, such studies have largely only targeted single cellular factors and are often extremely time-consuming. The few studies that have looked at CRISPR-mediated genetic manipulation in resting T cells are greatly limited due to cytotoxicity. In many cases, the cells do not survive beyond three days after transfection.

In this study, Dr. Albanese and his colleagues utilized optimized media conditions and the Lonza 4D-Nucleofector® System, which resulted in high cell survival of edited resting human CD4+ T cells. This optimized protocol, combined with the efficacy of Synthego's sgRNAs in generating highly efficient knockouts for both single and multiple genes, enabled the successful analysis of several genes that mediate HIV-1 infectivity in resting CD4+ T cells. Learnings from this study could be extended beyond virus-host cell interactions, including areas such as immuno-oncology and T cell biology.

To learn more about Synthego's CRISPRevolution products, go to Synthego.com/products/crispr-kits

To learn more about Lonza's 4D-Nucleofector® System, go to Bioscience.lonza.com/nucleofector-technology

> ***Our work is vital to the larger T cell community because now scientists can knock out genes in resting T cells and continue to maintain their resting states. This will allow scientists to study T cell activation factors. Our multiple gene knockout approach allows researchers to study T cell signaling pathways with redundant protein functions***
>
> *- Dr. Manuel Albanese*

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PROTOCOL

CRISPR Editing Human Primary Resting CD4+ T Cells with RNPs using Nucleofector® Technology

Developed by researchers at the University of Munich in collaboration with Synthego and Lonza

Introduction

This protocol describes how to deliver ribonucleoprotein (RNP) complexes that consist of purified SpCas9 nuclease complexed with Synthego's chemically modified synthetic single guide RNA (sgRNA) to human primary resting CD4+ T cells. RNP delivery is accomplished using the Lonza 4D-Nucleofector® System.

Chemically modified sgRNAs are designed to resist degradation by exonucleases and prevent innate intracellular immune cascades that can lead to cell death. The present protocol by Albanese et al. may be used for the generation of single or multiple gene knockouts in human primary resting CD4+ T cells[1]. This protocol was successfully used for the knock-in of GFP into different loci and may also be used for the knock-in of other genes of interest.

To learn more about the success of this protocol, please read the associated case study.

Abbreviations:

CRISPR: clustered regularly interspaced short palindromic repeats
SpCas9: *Streptococcus pyogenes* CRISPR-associated protein 9
sgRNA: single guide RNA
RNP: ribonucleoprotein
PCR: polymerase chain reaction
ICE: Inference of CRISPR Edits
TE: Tris-EDTA
PBS: phosphate-buffered saline
GFP: green fluorescent protein
FBS: fetal bovine serum
HDR: Homology-directed repair

Materials Required

Material	Ordering Information
sgRNA Synthetic sgRNA Kit (Synthego Standard Modifications)	(Synthego), available here
PCR & sequencing primers	Multiple vendors
Nuclease-free 1X TE buffer	Multiple vendors (e.g., Thermo Fisher Scientific, Catalog #AM9849)
Nuclease-free water	Multiple vendors (e.g, Thermo Fisher Scientific)
1X PBS, cell culture grade	Multiple vendors (e.g, Thermo Fisher Scientific)
4D-Nucleofector® Core Unit and X Unit	Lonza, 4D-Nucleofector® Core Unit | Lonza (AAF-1003B) Lonza, 4D-Nucleofector® X Unit | Lonza (AAF-1003X)
P3 Primary Cell 4D-Nucleofector® X Kit S (for 20 µl transfection volume)	(Lonza, P3 Primary Cell 4D-Nucleofector® X Kit S | Lonza (V4XP-3032) for 16x 20 µl transfection volume)
RPMI 1640 with GlutaMAX Supplement	Gibco RPMI 1640 Medium, GlutaMAX™ Supplement (thermofisher.com)
10% (v/v) FBS	(Sigma)
Penicillin/streptomycin (100 IU/ ml)	(Thermo Fisher Scientific)
EasySep™ Human CD4+ T Cell Enrichment Kit	(Stem Cell; 19052)
IL-7 and IL-15 (2 ng/ ml each)	(Peprotech)
SpCas9 Nuclease	Multiple vendors (IDT; 1081059)
Genomic Isolation Kit	Multiple vendors (e.g, Thermo Fisher Scientific)
CaCl2 (1mM)	Multiple vendors (e.g, Thermo Fisher Scientific)
MgCl2 (3mM)	Multiple vendors (e.g, Thermo Fisher Scientific)
EDTA (1mM)	Multiple vendors (e.g, Thermo Fisher Scientific)
Triton (X 100)	Multiple vendors (e.g, Thermo Fisher Scientific)
Tris (10 mM, pH 7.5)	Multiple vendors (e.g, Thermo Fisher Scientific)
Proteinase K	Multiple vendors (e.g, Thermo Fisher Scientific)
96-well flat-bottom plates	Multiple vendors (tissue culture grade)
48-well plates	Multiple vendors (tissue culture grade)
dsDNA HDR Template (optional)	Multiple vendors (e.g. Twist Bioscience)

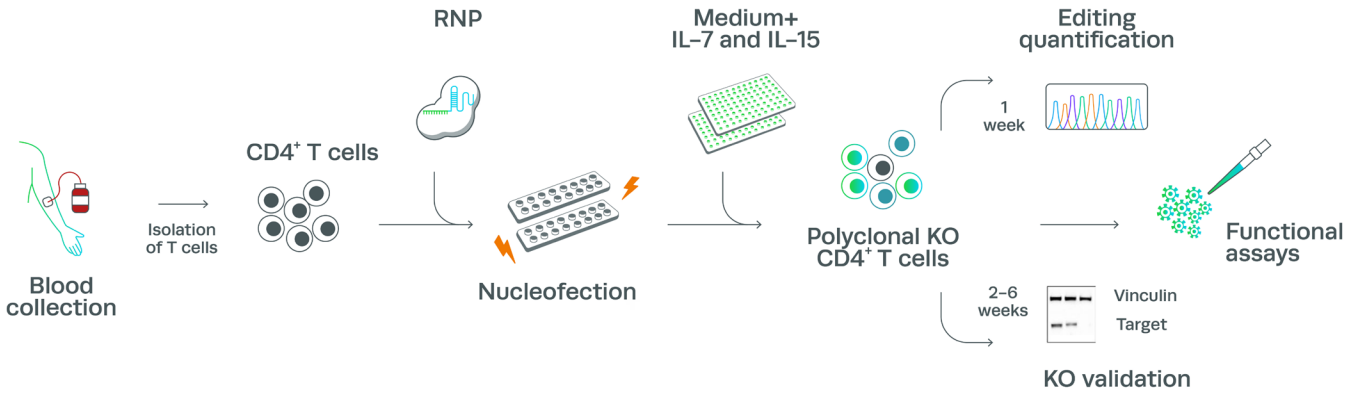
Note: All protocols outlined have been validated using materials mentioned in this manual. Materials other than the ones outlined in our manual may require additional optimization by the user.

General Guidelines

- Wearing gloves and using nuclease-free tubes and reagents is recommended to avoid RNase contamination.
- Always maintain sterile technique, and use sterile filter pipette tips.
- All Synthego and Lonza reagents should be stored according to the manufacturer's recommendations.
- Synthetic sgRNA should be dissolved in TE buffer and diluted to a working concentration using nuclease-free water.
- RNP complexes are stable at room temperature for up to 1 hour.
- This protocol uses 16-well Nucleocuvette® Strips for the 4D-Nucleofector® X Unit. Please note that Nucleofection® Conditions are transferable between different formats.

For further information regarding the above-described guidelines please check the Tips & Tricks section at the end of this protocol.

Workflow Graphic



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Protocol

1. Isolation of Primary T cells

- Heparinized blood retained in leukocyte reduction chambers from healthy donors was used to isolate CD4+ T cells.
- Blood cells were diluted with non-filter sterilized PBS; isolation of CD4+ T cells was done using the EasySep™ Rosette Human CD4+ T cell enrichment kits according to the manufacturer's protocols.
- For further information please check the Tips & Tricks section at the end of this protocol.

2. Pre Nucleofection®

A. Please make sure that the Nucleofector® Solution supplement is added to the P3 Nucleofector® Solution (16.4 µl P3 Solution + 3.6 µl supplement per sample).

B. Start 4D-Nucleofector® System and create experimental parameter file (for details see device manual).

C. Select the Nucleofector® Program EH-100. For other program recommendations, e.g., to achieve higher efficiency or higher viability, please see the Tips & Tricks at the end of this protocol.

D. Pre-warm an aliquot of culture medium RPMI 1640 GlutaMAX (without supplements) to 37°C (100 µl per sample); see Step 4 for recovery post Nucleofection®.

E. Prepare cell culture plates by filling the appropriate number of wells with the desired volume of recommended media.

3. Nucleofection® Process

3.1. Dissolve and Dilute your sgRNA

A. Briefly centrifuge your tubes containing the sgRNA to ensure that the dried RNA pellet is collected at the bottom.

B. If you are working with a 1.5 nmol sgRNA, rehydrate sgRNA in 15 µl nuclease-free buffer (1X TE buffer) and pulse vortex for 30 seconds to ensure complete mixing. This will make a stock solution of 100 µM (100 pmol/µl) of sgRNA.
Note: If sgRNA is not fully dissolved, let it sit overnight and up to 72 hours at 4°C. If the sgRNA is not being used immediately, dissolved sgRNA should be aliquoted into 6 µl per tube and stored at -20°C. Under these conditions, the sgRNA is stable for up to 3 years if not repeatedly thawed. For quantification guidelines and further information, please check the Tips & Tricks section at the end of this protocol.

C. The sgRNA 100 µM stock solution is ready to use. If you wish to dilute your sgRNA to a different working concentration, please use nuclease-free water, pulse vortex the sgRNA/nuclease-free water mix for 30 seconds and incubate at room temperature for 5 minutes to dissolve the sgRNA.

Option A: Knockout Generation in Resting Human CD4+ T Cells

This protocol describes amounts for one sample; multiple samples can be processed simultaneously if desired.

3.2. Assemble RNP Complex (2.5:1 sgRNA to SpCas9 ratio) and Transfect Cells

A. Prepare the RNP by incubating synthetic sgRNAs together with SpCas9 at a ratio of 2.5:1 to form the RNP complex as recommended in the table below.
Note: SpCas9 protein is provided in solution and can be used directly. Please use sterile filtered (0.22 µm) PBS to dilute the RNP to a final concentration of 20 µM.

RNP Preparation: Components, Concentration & Volume			
Component	**Quantity (pmol)**	**Concentration (µM)**	**Volume (µl)**
sgRNA	250	100	2.5
SpCas9*	100	62	1.6
PBS (filter sterilized)	N/A	N/A	0.9
Total Volume	N/A	N/A	5

*Please adjust volumes if a different SpCas9 concentration is used. The original protocol uses SpCas9 from IDT, alternatively, Synthego's 2NLS SpCas9 can also be used, available here.

B. Incubate RNP mix at room temperature for 10 minutes.

C. Count an aliquot of the cells and determine cell density.

D. Wash cells twice with non-filtered sterilized PBS, centrifuge 2 x 10^6 cells at 200 x g for 10 minutes at room temperature. Remove supernatant completely and resuspend the pellet carefully in 20 µl room temperature P3 Nucleofector® Solution.

E. Mix the RNP solution with the cell suspension as described in the table below depending on your editing method.

Nucleofection® Sample Preparation for Different Editing Methods		
Method	**Volume of RNP Solution**	**Cell Resuspension Volume in P3 Nucleofector® Solution**
Single sgRNA editing	5 µl of RNP	20 µl of cells
Multi-guide sgRNA editing	2 µl of each RNP complexes for each sgRNA	20 µl of cells
Co-editing of genes	0.5 µl of each RNP complexes for each sgRNA	20 µl of cells

F. Transfer 20 µl of the cells mixed with RNPs into a well of the 16-well Nucleocuvette® Strip.

Note: For example, for single sgRNA editing, mix 5 µl + 20 µl very well and only use 20 µl of sample for transfection.

G. Transfect cells using Nucleofector® Program EH-100.

H. Recover the cells according to Step 4.

Option B: Knock–in Generation in Resting Human CD4+ T Cells

This protocol describes amounts for one sample: multiple samples can be processed simultaneously if desired.

For the knock-in of GFP or any other gene of interest, a double-stranded DNA HDR donor template with around 550 bp in front 5' and 550bp after 3' the sgRNA cutting site is recommended.

3.3. *In vitro* digestion of knock–in HDR DNA templates to confirm no cleavage of the HDR template by the RNP (Optional)

A. Mix the RNP complex (1µM), the HDR DNA template (1µg) and single-cutter restriction enzyme for the specific plasmid (positive control of DNA cleavage).

B. Incubate samples at 37°C for 2h.

C. Add 1 µl of proteinase K (20 mg/ ml).

D. Incubate at 56°C for 10 minutes.

E. Visualize sample on a 1% agarose gel.

3.4. Assemble RNP Complex (2.5:1 sgRNA to SpCas9 Ratio) and Transfect Cells

A. Prepare the RNP by incubating synthetic sgRNAs together with SpCas9 at a ratio of 2.5:1 to form the RNP complex as recommended in the table below.
Note: SpCas9 protein is provided in solution and can be used directly. Please use sterile filtered (0.22 µm) PBS to dilute the RNP to a final concentration of 20 µM.

RNP and HDR Donor Preparation: Components, Concentration & Volume

Component	Quantity (pmol)	Concentration (µM)	Volume (µl)
sgRNA	250	100	2.5
SpCas9[1*]	100	62	1.6
PBS (filter sterilized)	N/A	N/A	0.9
Total Volume	N/A	N/A	5[1*]
DNA HDR donor template	0.8–1.12 pmol[2*] To be added after preparation of cell suspension		

[1*]Please adjust volumes if a different SpCas9 concentration is used. The original protocol uses SpCas9 from IDT, alternatively, Synthego's 2NLS SpCas9 can also be used, available here.
[2*]The pmol will vary with the DNA HDR donor template used. Usual templates span from 1900 bp to 1350 bp which correspond to the pmol above.

B. Count an aliquot of the cells and determine cell density.

C. Wash cells twice with non-filter sterilized PBS, centrifuge 2 x 10^6 cells at 200 x g for 10 minutes at room temperature. Remove supernatant completely and resuspend the pellet carefully in 20 µl room temperature P3 Nucleofector® Solution.

D. Mix 5 µl of the RNP template solution with 20 µl of the cell suspension.

E. Add donor DNA template (0.8-1.12 pmol).

F. Mix well and transfer 20 µl of the sample into a well of the 16-well Nucleocuvette® Strip.

G. Transfect cells using Nucleofector® Program EH-100.

H. Recover the cells according to Step 4.

4. Post Nucleofection®

A. Add 100 µl of pre-warmed RPMI 1640 GlutaMAX (without supplements) to each well in the 48-well plate.

B. Transfer cells to 48-well plates and allow them to recover for 10 min at 37 °C.

C. After recovery, cultivate and continue regular media changes until analysis as described in Step 5 below.

5. Cultivation of Primary Human CD4+ T Cells

A. Cultivate cells in 96-well flat-bottom plates at a cell density of 1×10^6 cells/ ml.

B. Add complete culture medium; RPMI 1640 GlutaMAX supplemented with 10% (v/v) FBS, penicillin/streptomycin (100 IU/ ml) and IL-7 and IL-15 (2 ng/ ml each) supplements.

C. Replace complete medium (including FBS, antibiotics and IL-7, IL-15 supplements) every 3 days.

6. Genotyping Analysis

Editing efficiencies can be assessed via Sanger sequencing through a variety of analysis tools. Synthego's ICE analysis tool can be used to assess both CRISPR knockout and knock-in edits.

To assess editing efficiency with ICE:

A. Design PCR primers compatible with Inference of CRISPR Edits (ICE) analysis.

B. 1 week post Nucleofection®, collect cells (5×10^4) and lyse in lysis buffer (20 µl) (1 mM CaCl2, 3 mM MgCl2, 1 mM EDTA, 1% Triton X 100 and 10 mM Tris, pH 7.5) with the addition of proteinase K (20 µg/ml; Thermo Fisher Scientific). Incubate the cell lysate at 65 °C for 20 min, followed by 95 °C for 15 min and store at −20°C until the PCR specific for the CRISPR/Cas9 target sites is performed.

C. Use 1 µl of cell lysate as a PCR template and Sanger-sequence the PCR amplicons.

D. Alternatively, you can follow the Genotyping guidelines provided in our Genotyping protocol for DNA isolation, PCR and Sanger sequencing.

E. Conduct Inference of CRISPR Edits (ICE) analysis on Sanger sequences to determine editing efficiency. See our ICE Knockout Analysis protocol or ICE Knock-in Analysis protocol for instructions on how to use ICE and interpret results.

Note: ICE should be able to handle any deletion sizes and work with any donor template size. However, Synthego has only validated ICE analysis of deletions up to 40 bases for sgRNA and up to 150 bases for multi-guide experiments. For knock-in experiments, we have tested insertions of up to 270 bases long.

The larger the deletion, the shorter the available Sanger sequence that will be used for the alignment with the control, hence the higher chance that ICE will display warning messages which are indicated when the analysis displays “Succeeded” in orange with the warning message below it. The Sanger sequence quality/ length will dictate if the analysis is successful.

Alternatively, if ICE analysis is not successful in genotyping your large knock-in edit please refer to loci-specific Sanger sequencing or functional assays to determine large knock-in success.

Tips & Tricks

- Cell preparation/ cell source

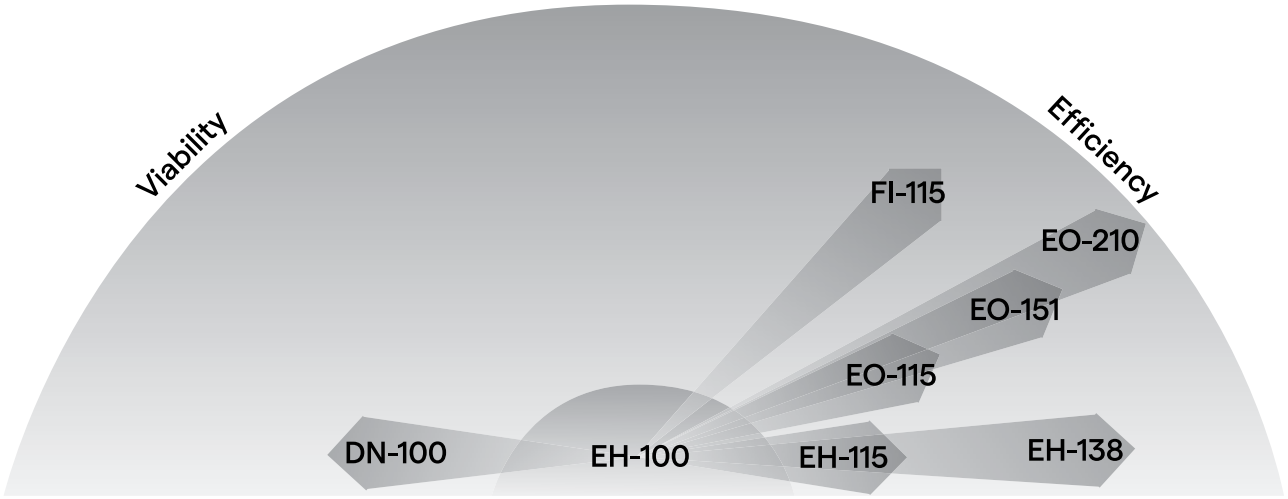
Alternatively to isolating primary T cells, cryopreserved PBMCs can be used to isolate CD4+ cells or cryopreserved CD4+ T cells can be used:

PBMCs (different fill sizes), cryopreserved	CC-2702 (50M), CC-2703 (100M), CC-2704 (10M), CC-2705 (25M)
CD4+ T cells, cryopreserved	2W-200

- Nucleofector® Program

The above protocol describes the use of pulse code EH-100 to transfect resting T-cells with Nucleofector® Technology;

Depending on your desired outcome (e.g. editing efficiency, number of viable transfected cells) you might want to test additional pulses. Please test (some of) the following pulse conditions along with a no pulse control:



The P3 Primary Cell 4D-Nucleofector® X Kit S (Lonza) is supplied with the pmaxGFP™ Vector as a positive control for the Nucleofection® Process. We recommend using 0.4 µg pmaxGFP™ Vector for a transfection volume of 20 µl.

- Viability

The centrifugation step prior to Nucleofection® Process highly influences the viability of transfected cells. Reducing centrifugation speed to 90 x g can improve cell viability.

To achieve maximum viability, we highly recommend adding pre-warmed media to each well of the Nucleocuvette® Strip directly after Nucleofection® and incubate for 10 minutes at room temperature. After that, carefully transfer the cells to a prepared culture dish in appropriate seeding density.

Medium change 6 hours post Nucleofection® may lead to increased viability after transfection. Spin the culture dishes for 8 minutes at 140 x g in a tissue culture centrifuge. Carefully remove the medium and add fresh pre-equilibrated culture medium as described in the protocol.

- Cell Numbers/ Throughput

Nucleofection® Conditions are transferable between different formats. The 4D-Nucleofector® X Unit offers the possibility to transfect in 20 µl format (described in this protocol) or in 100 µl format. For upscaling to 100 µl please adjust cell number and amount of RNP as shown in the table below:

	20 µl Volume	**100 µl Volume**
Kit #	P3 Primary Cell 4D-Nucleofector® X Kit S (V4XP-3032 for 16 x 20 µl reaction)	P3 Primary Cell 4D-Nucleofector® X Kit L (V4XP-3012 for 12 x 100 µl reaction; V4XP-3024 for 24 x 100 µl reaction)
Cell number	1–2 x 10^6	0.5–1 x 10^7
Nucleofector® Solution	20 µl (P3 + supplement)	100 µl (P3 + supplement)
SpCas9	100 pmol	500 pmol
sgRNAs	40 pmol	200 pmol
HDR template	0.8–1.12 pmol	4 – 5.6 pmol

In addition, it is possible to perform transfections in 20 µl volume in 96-well format using the 4D-Nucleofector® 96-well Unit or in 384-well format using the 384-well Nucleofector® System.

- Genome Editing in Other Cell Types

As a starting point, this protocol may also be used for activated T cells.

If you want to perform CRISPR Cas9-mediated genome editing or use a different editing system in other cell types (primary cells or cell lines), please check Lonza's Knowledge Center for detailed protocols and pulse recommendations or contact our Scientific Support Team.

Additionally, Synthego has other optimized RNP protocols available using synthetic sgRNAs for other cell types such as the Immortalized Cell Nucleofection Protocol and the iPS Cell Nucleofection Protocol.

- sgRNA and RNP

A. sgRNA Design

For knockouts, it is generally recommended to try 3-5 sgRNAs with unique target sequences (evaluated independently) per target that cut near (<10 bp) the edit site and check their indel percentage by ICE first. When using two sgRNAs together to create a knockout, the distance between the cutting sites must not be multiple of 3 nt. Otherwise, you remove only few amino acids and the protein can still be functional.

For knock-ins, the higher the indel efficiency of a given sgRNA, the higher the knock-in efficiency. Therefore, it is generally recommended to try 3-5 sgRNAs with unique target sequences (evaluated independently) per target that cut near (<10 bp) the edit site and check their indel percentage by ICE first.

B. sgRNA Rehydration, Storage, and Quantification

Synthetic sgRNA can also be dissolved in water if desired, however, we recommend using TE buffer to help stabilize pH when complexing with the nuclease. Please refer to our sgRNA Quick Start Guide and Gene Knockout Kit v2 Quick Start Guide for Rehydration, Storage, and Quantification guidelines.

C. RNP

It is important to work as quickly as possible as RNP complexes begin to become unstable after 1 hour at room temperature. Additionally, leaving cells in Nucleofector® Solution for extended periods of time may lead to reduced transfection efficiency and viability.

RNP complexes may also be stored at 4°C for up to one week, or at -20°C for up to one month. Note that RNPs stored at 4°C may become susceptible to contamination from microbial growth after long periods of time.

Dissolving RNPs in P3 Nucleofector® Solution is an option to avoid dilution of the Nucleofector® Solution. Nucleofector® Solution is animal component-free and non-toxic.

D. HDR Template

Our Tips and Tricks guide: Design and Optimization of CRISPR Knock-in Experiments offers general guidelines for planning your knock-in experiments.

The quality and the concentration of DNA HDR donor template used for Nucleofection® plays a central role for the efficiency of gene transfer. We strongly recommend the use of high-quality plasmids from vendors or, if isolated within the lab, use plasmid purification kits like EndoFree® Plasmid Kits (Qiagen, Cat. No. 12391 Giga Kit, 12362 Maxi Kit, 12381 Mega Kit). The purified DNA HDR donor template should be resuspended in deionized water or TE buffer with a concentration between 0.2-1 µg/µl. Please check the purity of each plasmid preparation by measuring the A260:A280 ratio, according to manufacturer's protocol. Other DNA donor templates could be used but will require optimization by the user.

If you wish to try alternative knock-in strategies, please be aware that we have no specific recommendations. There are limitations on the amount of donor DNA template you can use and the editing efficiency of each knock-in may vary.

Contact Technical Support

If you require further technical support, please reach out to our Scientific Support Teams:

- Synthego (support@synthego.com):
  - sgRNA and HDR template design, sgRNA resuspension
  - RNP formation
  - ICE analysis
- Lonza (Scientific.Support@lonza.com (US), Scientific.Support.EU@lonza.com (EU and International)):
  - T-cell preparation and cell handling
  - Nucleofection® Process, protocols and format

Citations

1. Albanese M et al. Rapid, efficient and activation-neutral gene editing of polyclonal primary human resting CD4+ T cells allows complex functional analyses. Nat Methods. 2022 Jan;19(1):81-89. doi: 10.1038/s41592-021-01328-8. Epub 2021 Dec 23. PMID: 34949807; PMCID: PMC8748193.

Additional Information

For an up-to-date list of all Synthego Protocols and other resources, please visit Synthego.com/resources

For technical assistance, contact our Scientific Support Team:

Ph: 888.611.6883 Email: support@synthego.com

About Synthego

Synthego is the leading genome engineering innovation company. The company's automated, full stack genome engineering platform enables broader access to CRISPR to accelerate basic scientific discovery, uncover cures for diseases, and develop novel synthetic biology applications. Headquartered in Silicon Valley, Synthego is used by scientists from the largest global biotechnology companies and global biology universities to unlock the potential of gene editing.

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