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Title: Improving CRISPR-Cas9 Screens in CAR T Cells: A Refined Method for Library Preparation

Authors and Affiliations:

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Author Questionnaire

- 1. Microscopy:** Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **No**
- 2. Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **No.**
- 3. Filming location:** Will the filming need to take place in multiple locations? **Yes. The location is CIMA - Universidad de Navarra building and due to the equipment needed for the protocol, the filming need to take place in different labs located in the same floor**
- 4. Testimonials (optional):** Would you be open to filming two short testimonial statements **live during your JoVE shoot**? These will **not appear in your JoVE video** but may be used in JoVE's promotional materials. **Yes.**

Current Protocol Length

Number of Steps: 25

Number of Shots: 56

Introduction

Videographer: Obtain headshots for all authors available at the filming location.

INTRODUCTION:

REQUIRED: Summarize your research focus and the main questions it aims to answer.

- 1.1. **Maider Garnica:** Our research focuses on using CRISPR screening approaches to identify targets that enhance the effectiveness of CAR T cell therapies.
 - 1.1.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

~~What are the current experimental challenges?~~

- 1.2. **Maider Garnica:** Current experimental challenges include achieving reproducible library preparation methods in CRISPR screening approaches.
 - 1.2.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

CONCLUSION:

~~What advantage does your protocol offer compared to other techniques?~~

- 1.3. **Maider Garnica:** Our protocol offers a reliable alternative to existing library preparation techniques used in CRISPR screening approaches.
 - 1.3.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

~~How will your findings advance research in your field?~~

- 1.4. **Maider Garnica:** Our findings will advance the field by supporting the development of more reproducible and robust CRISPR screening protocols.
 - 1.4.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

~~What new scientific questions have your results paved the way for?~~

1.5. **Maider Garnica:** Our results pave the way for identifying genes that can be modulated to enhance CAR T cell phenotype and function.

1.5.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

Videographer: Obtain headshots for all authors available at the filming location.

Testimonial Questions (OPTIONAL):

Videographer: Please capture all testimonial shots in a wide-angle format with sufficient headspace, as the final videos will be rendered in a 1:1 aspect ratio. Testimonial statements will be presented live by the authors, sharing their spontaneous perspectives.

- Testimonial statements will **not appear in the video** but may be featured in the journal's promotional materials.
- **Provide the full name and position** (e.g., Director of [Institute Name], Senior Researcher [University Name], etc.) of the author delivering the testimonial.
- Please **answer the testimonial question live during the shoot**, speaking naturally and in your own words in **complete sentences**.

AUTHORS: Please note a change in the testimonial questions

What is the key takeaway you hope viewers remember after watching your video article?

- 1.6. **Maider Garnica, PhD Student, CIMA Universidad de Navarra**: (authors will present their testimonial statements live)
 - 1.6.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

What value do you expect this video article to have for other researchers working in your field?

- 1.7. **Maider Garnica, PhD Student, CIMA Universidad de Navarra** : (authors will present their testimonial statements live)
 - 1.7.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

Ethics Title Card

This research has been approved by the Institutional Review Board (IRB) at the Universidad de Navarra

Protocol

2. CAR T Cell Production and CRISPR-Cas9 Screening Workflow in Primary Human T Cells

Demonstrator: Maider Garnica

AUTHORS: Please note that all pronunciation guides are given in red, italics. Kindly go through the same and change where necessary.

- 2.1. To begin, add a predetermined amount of lentiviral CRISPR (*Krisper*) library to a 24-well plate containing T-cells [1]. Add polybrene at a final concentration of 8 micrograms per milliliter [2]. Mix gently to ensure even distribution [3].
 - 2.1.1. Talent pipetting the lentiviral CRISPR library to the cell suspension.
 - 2.1.2. Talent adding polybrene to the well. **NOTE: 2.1.2 and 2.1.3 were combined**
 - 2.1.3. Talent gently mixing the contents.
- 2.2. Centrifuge the cells at 700 *g* for 90 minutes at 32 degrees Celsius to perform spinfection [1]. After centrifugation, carefully remove the plate from the centrifuge [2].
 - 2.2.1. Talent placing the plate into the centrifuge and setting the parameters.
 - 2.2.2. Talent removing the plate from the centrifuge after completion.
- 2.3. Replace the media with fresh T cell growth medium [1].
 - 2.3.1. Talent aspirating old media and adding fresh T cell growth medium.
AND
TEXT ON PLAIN BACKGROUND:
T-cell Growth Medium:
3% HS
1% P/S
IL-7: 625 IU/mL
IL-15 : 85 IU/mL
Final cell density: 10⁶ cells/mL
Video Editor: Please play both shots side by side in a split screen
- 2.4. After 6 hours, add the CAR (*car*) lentiviral vector at a multiplicity of infection of 3 [1]. Incubate the cells at 37 degrees Celsius for 4 days [2].
 - 2.4.1. Talent pipetting CAR lentiviral vector to the culture.
 - 2.4.2. Talent placing the culture into the incubator.
- 2.5. Once incubation is complete, collect the CAR T-cells [1]. Wash once with PBS [2]. Then stain the cells using acridine orange propidium iodide [3] and count them in a

fluorescent cell counter [4].

- 2.5.1. Shot of CAR T cell pellet in a tube.
- 2.5.2. Talent adding phosphate-buffered saline to the tube with cell pellet.
- 2.5.3. Talent adding acridine orange propidium iodide to the cells.
- 2.5.4. Shot of the stained cell suspension being placed in a fluorescent cell counter.
- 2.6. Now, prepare a 4 micromolar solution of Cas9 (*Kass-Nine*) in electroporation buffer [1].
 - 2.6.1. Talent adding Cas9 to electroporation buffer.
- 2.7. Resuspend the CAR T cells in the Cas9 solution [1-TXT]. Then pipette 50 microliters of the cell suspension into each well of a multiwell cuvette strip without forming bubbles [2]. Remove any visible bubbles by gently running a pipette tip along the edges of the well [3].
 - 2.7.1. Talent adding 50 μ L of Cas9 solution to the cell suspension. **TXT: 50 μ L per 5 x 10⁶ cells**
 - 2.7.2. Talent loading 50 μ L of cell suspension into the wells of a multiwell cuvette strip.
 - 2.7.3. Talent removing bubbles with a pipette tip.
- 2.8. Use the **EXPAND T CELL 3** program to nucleofect the CAR T cells [1]. Place the cuvettes in an incubator at 37 degrees Celsius for 40 minutes [2].
 - 2.8.1. Talent selecting the EXPAND T CELL 3 program.
Videographer: Please film the screen of the instrument for this step
 - 2.8.2. Talent placing cuvettes into the incubator.
- 2.9. Resuspend the nucleofected cells in T cell growth medium [1]. Then incubate again at 37 degrees Celsius for 3 days [2].
 - 2.9.1. Talent adding growth medium to nucleofected cells.
 - 2.9.2. Talent placing cells back into the incubator.
- 2.10. Count the cells using acridine orange propidium iodide staining in a fluorescent cell counter [1]. ~~Now, adjust the cell concentration to 10⁶ cells per milliliter using T cell growth medium containing interleukin 7 and interleukin 15 [2].~~ After adjusting the cell density to 10⁶ cells per milliliter, add puromycin to a final concentration of 2.5 micrograms per milliliter [3-TXT].
 - 2.10.1. Talent counting stained cells using the fluorescent cell counter.
 - 2.10.2. ~~Talent diluting or concentrating cells to the desired density.~~ **NOTE: Not filmed**
 - 2.10.3. Talent adding puromycin to the culture. **TXT: Count and readjust cells every 2 days to 10⁶ cells/mL using growth medium with puromycin**
- 2.11. After 6 days, assess the percentage of CAR-positive cells using flow cytometry [1]. ~~Then~~

~~sort CAR T cell populations [2] and store dry cell pellets at minus 80 degrees Celsius [3].~~

2.11.1. Talent placing the cell suspension into a flow cytometer.

~~2.11.2. Shot of tubes with sorted CAR T cells.~~ **NOTE: Not filmed**

~~2.11.3. Talent placing the dried cell pellets at 80 °C freezer.~~

3. Genomic DNA Digestion and sgRNA Cassette Enrichment for CRISPR Screening

3.1. Extract genomic DNA from the samples using a cell and tissue DNA extraction method [1-TXT]. Measure genomic DNA concentration using a double-stranded DNA quantification kit [2].

3.1.1. Shot of extracted gDNA in labeled tubes. **TXT: Elute twice for maximum recovery**

3.1.2. Talent measuring DNA concentration in a spectrophotometer.

3.2. Now, digest up to 5 micrograms of genomic DNA with 20 units of the selected restriction enzymes in a total volume of 50 microliters [1]. Incubate overnight at 37 degrees Celsius in a thermocycler [2].

3.2.1. Talent adding restriction enzymes to 5 µg of gDNA. **NOTE: 3.2.1.1. shows Close up adding restriction enzyme**

3.2.2. Talent placing tubes into a thermocycler.

3.3. Then next day, prepare 10 milliliters of Elution buffer and 50 milliliters of fresh 70% ethanol [1]. Pipette 100 microliters of the digested product into a tube containing vortexed beads [2].

3.3.1. Shot of prepared Elution buffer and ethanol solutions. **TXT: Elution Buffer: 10 mM Tris-HCl, pH 8.0**

3.3.2. Talent pipetting 100 µL of the digested product into a tube with vortexed beads.

3.4. Pipette to mix the suspension thoroughly then incubate [1-TXT] before magnetizing for another 5 minutes [2].

3.4.1. Talent pipetting the suspension up and down. **TXT: Incubation: RT, 5 min**

3.4.2. Talent placing the tube onto a magnet. **NOTE: 3.4.1 and 3.4.2. were combined**

3.5. While on the magnet, pipette 200 microliters of the 70% ethanol solution without disturbing the pellet [1]. After 30 seconds, discard the supernatant [2-TXT].

3.5.1. Shot of 200 µL of 70% ethanol being pipetted into the tube.

3.5.2. Talent pipetting out the supernatant. **TXT: Perform magnetization and alcohol wash 2x**

3.6. After 2 washes, allow the beads to dry for 2 minutes [1]. Then remove them from the magnet [2], add 40 microliters of elution buffer [3] and pipette to resuspend the beads [4-TXT].

3.6.1. Shot of dried beads.

3.6.2. Talent removing the beads from the magnet. **NOTE: 3.6.2, 3.6.3, 3.6.4 were combined**

3.6.3. Talent pipetting 40 μ L of elution buffer.

3.6.4. Shot of the suspension being pipetted to resuspend the beads.

3.7. Magnetize the suspension for 2 minutes [1] then transfer the supernatant into a new tube [2].

3.7.1. Talent placing the suspension on the magnet.

3.7.2. Shot of the supernatant being pipetted into a new tube.

4. Streptavidin-Based Pulldown of sgRNA Cassettes for CRISPR-Cas9 Screening

4.1. Add 1x Wash/Binding (*wash-binding*) Buffer to a tube containing 1 milligram of vortexed streptavidin magnetic beads [1]. Pipette to resuspend the beads in the Buffer [2].

4.1.1. Talent adding wash/binding buffer to a tube with vortexed streptavidin magnetic beads.

AND

TEXT ON PLAIN BACKGROUND:

Wash/Binding Buffer 2x:

10 mM Tris-HCl, pH 7.5

2 M NaCl

1 mM EDTA

Dilute half of the volume with H₂O to 1x

Video Editor: Please play both shots side by side in a split screen

4.1.2. Talent resuspending beads in buffer.

4.2. Then place the suspension on a magnet for 1 minute [1] before discarding the supernatant [2-TXT].

4.2.1. Shot of the suspension being placed on a magnet.

4.2.2. Shot of the supernatant being discarded. **TXT: Perform wash 3x**

4.3. After the third wash, resuspend the beads in 2x Wash/binding buffer [1]. Then Add 5 microliters of 10 micromolar biotinylated pulldown primers to the purified digestion product [2].

4.3.1. Shot of the beads being resuspended in 2x wash/binding buffer.

- 4.3.2. Talent adding 5 μ L of 10 μ M biotinylated pulldown primers to the purified digestion product
- 4.4. Incubate the mixture in a dry bath at 96 degrees Celsius for 5 minutes [1] and immediately cool on ice for 5 minutes [2].
 - 4.4.1. Talent placing the mixture in a dry bath at 96°C. **NOTE: 4.4.1 and 4.4.2 were combined**
 - 4.4.2. Shot of the tubes being plunged into ice.
- 4.5. Now add 1 milligram of the pre-washed streptavidin magnetic beads to the reaction [1]. Incubate for 20 minutes at room temperature while resuspending every 4 minutes [2].
 - 4.5.1. Talent adding beads to the sample. **NOTE: 4.5.1 and 4.5.2 were combined**
 - 4.5.2. Talent gently mixing the sample during incubation.
- 4.6. Magnetize the beads for 5 minutes [1]. Then wash three times with 1x Wash/Binding Buffer [2].
 - 4.6.1. Talent placing the beads on a magnetic rack.
 - 4.6.2. Shot of 1x wash/binding buffer being added to the tube.
- 4.7. Finally, resuspend the beads containing bound sgRNA cassettes in 50 microliters of elution buffer [1]. Prepare and amplify the sgRNA library and sequence it [2], then process sequencing data to extract, align, and analyze sgRNA counts for candidate gene identification [3].
 - 4.7.1. Talent resuspending beads in 50 μ L elution buffer.
 - 4.7.2. *Reuse 3.2.2* **NOTE: Added at post-shoot stage**
 - 4.7.3. *Reuse 3.4.1*

Results

5. Results

- 5.1. Following antibiotic selection, CAR T cells transduced with the CRISPR (*crisper*) library showed continued survival and proliferation in both donors after puromycin treatment [1].
 - 5.1.1. LAB MEDIA: Figure 1B. *Video editor: Highlight the brown puromycin bars in both donor panels.*
- 5.2. Untransduced T cells and CAR T cells lacking CRISPR library transduction did not show comparable proliferation under antibiotic selection and served as negative controls [1].
 - 5.2.1. LAB MEDIA: Figure 1B. *Video editor: Highlight the UTD and CAR bars (grey) without puromycin in both donor panels.*
- 5.3. Restriction enzyme digestion with NdeI (*N-del*) and PspXI (*P-S-P-X-I*) generated fragmented genomic DNA while preserving the single guide RNA cassette for enrichment [1]. Fragments containing sgRNA were then selectively amplified [2].
 - 5.3.1. LAB MEDIA: Figure 2C. *Video editor: Highlight the digested genomic DNA lanes compared*
 - 5.3.2. LAB MEDIA: Figure 2D.
- 5.4. Pearson and Spearman correlations showed that single guide RNA read counts and library complexity were maintained in donor genomic DNA samples compared with the plasmid library [1].
 - 5.4.1. LAB MEDIA: Figure 3C. *Video editor: Sequentially Highlight the scatter plots*
- 5.5. Robust rank aggregation analysis identified genes that were significantly enriched or depleted following Cas9 editing in both donors [1].
 - 5.5.1. LAB MEDIA: Figure 3D and E. Video editor: Sequentially show D and then E
- 5.6. Pathway enrichment analysis revealed top Gene Ontology terms associated with the identified genetic regulators [1].
 - 5.6.1. LAB MEDIA: Figure 3F.

1. Lentiviral

Pronunciation link: <https://www.merriam-webster.com/dictionary/lentiviral>

- IPA: /ˌlen.tʃiˈvaɪ.rəl/
Phonetic Spelling: len·tee·vy·ruhl
2. CRISPR
Pronunciation link: <https://www.merriam-webster.com/dictionary/CRISPR>
IPA: /ˈkris.pə/
Phonetic Spelling: kris·per
 3. Polybrene
Pronunciation link: <https://www.howtopronounce.com/polybrene>
IPA: /ˈpɑː.li.briːn/
Phonetic Spelling: paa·lee·breen
 4. Centrifuge
Pronunciation link: <https://www.merriam-webster.com/dictionary/centrifuge>
IPA: /ˈsen.trə.fjuːdʒ/
Phonetic Spelling: sen·truh·fyooj
 5. Spinfection
Pronunciation link: No confirmed link found
IPA: /spɪnˈfek.ʃən/
Phonetic Spelling: spin·fek·shuhn
 6. Multiplicity
Pronunciation link: <https://www.merriam-webster.com/dictionary/multiplicity>
IPA: /ˌmʌl.təˈplɪs.ə.ti/
Phonetic Spelling: mul·tuh·pliss·uh·tee
 7. Acridine
Pronunciation link: <https://www.merriam-webster.com/dictionary/acridine>
IPA: /ˈæk.rɪ.diːn/
Phonetic Spelling: ak·ruh·deen
 8. Propidium
Pronunciation link: <https://www.howtopronounce.com/propidium>
IPA: /proʊˈpɪd.i.əm/
Phonetic Spelling: proh·pid·ee·uhm
 9. Fluorescent
Pronunciation link: <https://www.merriam-webster.com/dictionary/fluorescent>
IPA: /flʊˈres.ənt/
Phonetic Spelling: floor·ess·uhnt
 10. Electroporation
Pronunciation link: <https://www.merriam-webster.com/dictionary/electroporation>
IPA: /ɪˌlek.troʊ.pəˈreɪ.ʃən/
Phonetic Spelling: ih·lek·troh·puh·ray·shuhn
 11. Cuvette
Pronunciation link: <https://www.merriam-webster.com/dictionary/cuvette>
IPA: /kjuːˈvet/
Phonetic Spelling: kyoo·vet
 12. Nucleofect
Pronunciation link: No confirmed link found

- IPA: /'nu:.kli.oo.fekt/
Phonetic Spelling: noo·klee·oh·fekt
13. Interleukin
Pronunciation link: <https://www.merriam-webster.com/dictionary/interleukin>
IPA: /,ɪn.tə.'lu:.kɪn/
Phonetic Spelling: in·ter·loo·kɪn
14. Puromycin
Pronunciation link: <https://www.merriam-webster.com/dictionary/puromycin>
IPA: /,pju:.roo'maɪ.sɪn/
Phonetic Spelling: pyoo·roh·my·sɪn
15. Cytometry
Pronunciation link: <https://www.merriam-webster.com/dictionary/cytometry>
IPA: /saɪ'tæ:.mə.tri/
Phonetic Spelling: sy·tom·uh·tree
16. Genomic
Pronunciation link: <https://www.merriam-webster.com/dictionary/genomic>
IPA: /dʒə'noʊ.mɪk/
Phonetic Spelling: juh·noh·mɪk
17. Spectrophotometer
Pronunciation link: <https://www.merriam-webster.com/dictionary/spectrophotometer>
IPA: /,spek.troʊ.fə'tæ:.mə.tə/
Phonetic Spelling: spek·troh·fuh·taa·muh·ter
18. Thermocycler
Pronunciation link: <https://www.howtopronounce.com/thermocycler>
IPA: /'θɜ:.moʊ.sai.klə/
Phonetic Spelling: thur·moh·sy·kler
19. Streptavidin
Pronunciation link: <https://www.merriam-webster.com/dictionary/streptavidin>
IPA: /,strep.tə'vaɪ.dɪn/
Phonetic Spelling: strep·tuh·vy·dɪn
20. Biotinylated
Pronunciation link: <https://www.howtopronounce.com/biotinylated>
IPA: /baɪ'ou.tɪ.nə.leɪ.tɪd/
Phonetic Spelling: by·oh·tin·uh·lay·tid
21. Magnetize
Pronunciation link: <https://www.merriam-webster.com/dictionary/magnetize>
IPA: /'mæɡ.nə.taɪz/
Phonetic Spelling: mag·nuh·tyze
22. Supernatant
Pronunciation link: <https://www.merriam-webster.com/dictionary/supernatant>
IPA: /'su:.pə'nei.tənt/
Phonetic Spelling: soo·per·nay·tuhnt
23. Elution
Pronunciation link: <https://www.merriam-webster.com/dictionary/elution>

IPA: /i'lu:.ʃən/

Phonetic Spelling: ee·loo·shuhn

24. Correlation

Pronunciation link: <https://www.merriam-webster.com/dictionary/correlation>

IPA: /,kə:r.ə'leɪ.ʃən/

Phonetic Spelling: kor·uh·lay·shuhn

25. Spearman

Pronunciation link: <https://www.merriam-webster.com/dictionary/Spearman>

IPA: /'spɪr.mən/

Phonetic Spelling: speer·muhn

26. Pearson

Pronunciation link: <https://www.merriam-webster.com/dictionary/Pearson>

IPA: /'pɪr.sən/

Phonetic Spelling: peer·suhn

27. Aggregation

Pronunciation link: <https://www.merriam-webster.com/dictionary/aggregation>

IPA: /,æg.rɪ'geɪ.ʃən/

Phonetic Spelling: ag·ruh·gay·shuhn

28. Ontology

Pronunciation link: <https://www.merriam-webster.com/dictionary/ontology>

IPA: /ɑ:n'tɑ:.lə.dʒi/

Phonetic Spelling: aan·taa·luh·jee