FINAL SCRIPT: APPROVED FOR FILMING

Submission ID #: 62299
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Project Page Link: https://www.jove.com/account/file-uploader?src=19001243

Title: Production of human CRISPR-engineered CAR-T cells

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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? **Yes**

3. **Interview statements:** Considering the COVID-19-imposed mask-wearing and social distancing recommendations, which interview statement filming option is the most appropriate for your group? **Please select one**.

   ☒ Interviewees wear masks until videographer steps away (≥6 ft/2 m) and begins filming, then the interviewee removes the mask for line delivery only. When take is captured, the interviewee puts the mask back on. Statements can be filmed outside if weather permits.

Videographer: Film the screen as a backup for all SCREEN shots

4. **Filming location:** Will the filming need to take place in multiple locations? **No**

Current Protocol Length

Number of Steps: 09
Number of Shots: 14

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Carl June:** Our protocol enables the combination of human genome engineering with CAR T cell therapy. With this approach it is possible to achieve disruption of up to three genes in cells at high efficiency, and to produce clinical grade human T cells using multiplex CRISPR-Cas9 engineering.

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

1.2. **Bruce Levine:** The methods described can be applied to other CAR constructs and target genes. The basis of the techniques is robust enough to be translated to larger scale and to academic and industry collaborators for further development.

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

OPTIONAL:

1.3. **SA/NW:** When attempting this protocol for the first time, limit the number of groups in gRNA screens to enable accurate incubation times. Adhering to culturing conditions and seeding density as described have proven to be critical in our experience.

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

Commented [AG1]: Authors: Please make a note of who delivers this statement during the shoot

Protocol

2. Designing of sgRNAs and Gene Disruption in Primary Human T cells

2.1. To begin, obtain autologous peripheral blood mononuclear cells or PBMCs from healthy volunteer donors and isolate CD4 and CD8 positive T-cells using commercially available CD4 and CD8 selection kits **[1]**.

2.1.1. WIDE: Talent working on a lab bench. **NOTE: Use File 5Y7A2489.MP4.**

2.2. Combine CD4 plus and CD8 plus T-cells in a 1 to 1 ratio **[1]** and incubate 3 million cells per milliliter in R10 supplemented with 5 nanograms per milliliter of each human IL-7 and human IL-15 overnight at 37 degrees Celsius **[2].**

2.2.1. Talent combing the CD4 and CD8 cells together. **NOTE: Video starts from here.**

2.2.2. Talent incubating the tube. **NOTE: 2 separate shots; 2.2.2., and 2.2.3.**

2.3. On the next day, count the T-cells and centrifuge 5 to 10 million cells at 300 times g for 5 minutes **[1]**. Discard all supernatant **[2]** and wash the cell pellet in reduced-serum minimal essential media **[3]**. Resuspend the pellet in 100 microliters of nucleofection solution according to the manufacturer's instructions **[4]**.

2.3.1. Talent putting tubes for centrifugation

2.3.2. Talent removing supernatant. **NOTE: Use take 2.**

2.3.3. Talent washing the pellet. **NOTE: 2 separate shots; 2.3.3., and 2.3.3. additional.**

2.3.4. Talent resuspending the pellet

2.4. While washing the cells, prepare ribonucleoprotein, or RNP, complex by incubating 10 micrograms of Cas9 *(pronounce: "kas nine")* nuclease with 5 micrograms of single guide RNA for 10 minutes at room temperature. Include a mock control without sgRNA **[1].**

2.4.1. Talent combining Cas9 and sgRNA **TEXT: 1:2.4 molar ratio of Cas9 to sgRNA** *Videographer: This step is important!* **NOTE: Take 2 is a close-up of just one section of step 2.4.1**

2.5. Combine the resuspended cells with the RNP complex and add 4.2 microliters of 4 micromolar electroporation enhancer **[1-TXT]**. Mix well and transfer into electroporation cuvettes **[2]**. **NOTE: Shot 3 separate shots; 2.5.0, 2.5.1., and 2.5.2.**

2.5.1. Talent adding electroporation enhancer to the cells **TEXT: Enhancer: ssDNA oligonucleotide; non-homologous to human genome** *Videographer: This step is difficult and important!*

2.5.2. Talent transferring the mixture into electroporation cuvettes *Videographer: This step is difficult and important!* **NOTE: Take 2 is a close-up of liquid going into the cuvette which they thought would be helpful.**

2.6. Electroporate the cells using pulse code EH111 **[1]**, then incubate 5 million cells per milliliter in R10 supplemented with 5 nanograms per milliliter human IL-7 and human IL-15 at 30 degrees Celsius for 48 hours in 12-well plates. After the incubation, proceed with T-cell activation and expansion **[2]**. **NOTE: Broken down into 4 steps; 2.6.1.-2.6.4.**

2.6.1. Talent electroporating the cells

2.6.2. Talent incubating 12-well plate

3. CRISPR Efficiency: Sequencing and Indel Detection

3.1. Design sequencing primers by entering the sequence of the PCR amplicon into a standard primer design software. The design software will suggest multiple primer sequences that are suitable for Sanger sequencing **[1]**.

3.1.1. SCREEN: Talent entering the PCR amplicon sequence in the software and primer sequences generated. **NOTE: Use file 3.1.1_Jove.mov from 00:01-00:10 & 00:32-00:35.** *Video Editor: Speedup the video and play.*

3.2. Choose forward and reverse primers that bind within the amplicon at least 150 base pairs upstream or downstream of the gRNA cut site to ensure good sequencing quality around the indels **[1]**.

3.2.1. SCREEN: Talent choosing the appropriate sequence **NOTE: Use file 3.2.1_Jove.mov from 00:01-0:41.** *Video Editor: Speedup the video and play.*

3.3. Use TIDE analysis to detect knock out efficiency at the genomic level. The algorithm accurately reconstructs the spectrum of indels from the sequence traces and calculates R square values **[1-TXT].**

3.3.1. SCREEN: Talent using performing TIDE analysis **TEXT: TIDE: Tracking of Indels by Decomposition. NOTE: Use file 3.3.1_Jove.mov from 00:01-00:19.** *Video Editor: Speedup the video and play.*

Results

4. Results: Manufacturing Human CAR-T cells using CRISPR Cas9 Edited T cells

4.1. After activation, T cells are expanded in culture and cryopreserved for future studies. During the expansion, the population doubling and volume changes are tracked throughout the protocol for both mock and edited CAR-T cells. No significant changes were detected in the proliferation and activation during the expansion **[1].**

4.1.1. LAB MEDIA: Figure 2B and 2C

4.2. Once the cells were cryopreserved, levels of CAR expression were determined for further functional studies for both the Mock edited and knock out CAR-T cells. No significant changes were observed **[1].**

4.2.1. LAB MEDIA: Figure 2D

4.3. Knock out efficiency can be determined using multiple techniques. In the representative flow cytometry plots, PDCD1 and TRAC locus was targeted using sgRNA, showing an efficiency of 90% for the PDCD1 sgRNA and 98% for the TRAC sgRNA across multiple healthy donors **[1].**

4.3.1. LAB MEDIA: Figure 3

Conclusion

5. Conclusion Interview Statements

5.1. **SA/NW:** It is important to make sure the molar ratios of Cas9 and gRNA are accurate. Also, the cells should always be at 4 degrees Celsius and not be left in the electroporation solution for extended periods of time.

Commented [AG2]: Authors: Please make a note of who delivers this and the next statement during the shoot

5.1.1. INTERVIEW: Named talent says the statement above in an interview style shot, looking slightly off-camera. *Suggested B-roll: 2.5 and 2.6*

5.2. **SA/NW:** After cryopreservation, the CRISPR engineered CAR T cells can be used for in vitro and in vivo functional assays such as cytotoxicity assays, cytokine production and syngeneic or xenograft tumor mouse models.

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

5.3. **Carl June:** This approach has paved the way for a number of experiments that are currently on going on a global scale. Multiple clinical trials are now being conducted using ex vivo CRISPR-Cas9 engineering for cancer and for nonmalignant genetic disorders such as the hemoglobinopathies.

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