Submission ID #: 61855
Scriptwriter Name: Anastasia Gomez
Project Page Link: https://www.jove.com/account/file-uploader?src=18865823

Title: Genome Engineering of Primary Human B Cells Using CRISPR/Cas9

Authors and Affiliations:

Kanut Loaharawee[1,2,3], Matthew J. Johnson[1,2,3], Walker S. Lahr[1,2,3], Joseph J. Peterson[1,2,3], Beau R. Webber[1,2,3], Branden S. Moriarity[1,2,3]

[1]Department of Pediatrics, University of Minnesota, Minneapolis MN, USA
[2]Center for Genomic Engineering, University of Minnesota, Minneapolis MN, USA
[3]Masonic Cancer Center, University of Minnesota, Minneapolis MN, USA

Corresponding Authors:

Branden S. Moriarity (mori0164@umn.edu)

Email Addresses for All Authors:

laoha002@umn.edu
joh02946@umn.edu
wslahr@umn.edu
pet01059@umn.edu
webb0178@umn.edu
mori0164@umn.edu

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. **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 self-record interview statements. JoVE can provide support for this option.

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

Current Protocol Length

Number of Steps: 13
Number of Shots: 39

Introduction

1. Introductory Interview Statements

NOTE: Author self-recorded all interview statements and uploaded everything to the project page (audio and video).

REQUIRED:

1.1. **Kanut Loaharawee:** This protocol allows researchers to precisely eliminate a gene in B cells to study the loss of function effects of that gene, or to insert a transgene in B cells for stable transgene expression that can be used for research or therapeutic purposes.

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

1.2. **Kanut Loaharawee:** The main advantage of this method is that it provides a universal platform that makes it possible to easily and precisely engineer B cells with high efficiencies.

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

OPTIONAL:

1.3. **Kanut Loaharawee:** This method can be used to engineer B cells to express recombinant antibodies or enzymes and then transfer those B cells to treat infections or enzymopathies.

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

Ethics Title Card

1.4. Procedures involving human subjects have been approved by the Institutional Review Board (IRB) at the University of Minnesota.

Protocol

2. Human B-cell Expansion

2.1. To begin, thaw B cells in a 37-degree Celsius water bath **[1]**. While waiting, transfer 2 milliliters of pre-warmed FBS into a sterile 15-milliliter conical tube **[2]**. Once the B cells are completely thawed, immediately add 1 milliliter of pre-warmed FBS, drop-wise, into the sample. Incubate at room temperature for 1 minute **[3]**.

2.1.1. WIDE: Establishing shot of talent placing cells in a water bath.

2.1.2. Talent transferring FBS to a tube.

2.1.3. Talent adding FBS to the cells.

2.2. Gently pipette to resuspend the sample **[1]** and transfer the whole volume, dropwise, into a conical tube containing 2 milliliters of pre-warmed FBS **[2]**. Bring the volume to 15 milliliters with sterile PBS **[3]**, then cap the tube and invert it gently 2 to 3 times **[4]**. *Videographer: This step is important!*

2.2.1. Talent resuspending the sample.

2.2.2. Talent transferring the sample to a conical tube.

2.2.3. Talent adding PBS to the tube.

2.2.4. Talent capping the tube and inverting it.

2.3. Centrifuge the sample at 400 × *g* for 5 minutes **[1]**, then discard the supernatant without disturbing the cell pellet **[2]**, resuspend the pellet with 1 milliliter of pre-equilibrated B-cell expansion medium **[3]**, and count the cells. The total cell number should be approximately 10^7 cells **[4]**.

2.3.1. Talent putting the tube in the centrifuge and closing the lid.

2.3.2. Talent discarding the supernatant. Vid NOTE: Use the 2nd part of Take 1

2.3.3. Talent resuspending the cells.

2.3.4. Talent counting the cells.

2.4. Transfer the cells into a flask containing 20 milliliters of the pre-equilibrated B-cell expansion medium **[1]**. The final concentration of the cells should be approximately 5 x 10^5 cells per milliliter. Incubate the flask vertically in a tissue-culture incubator **[2]**.

2.4.1. Talent transferring the cells into the flask.

2.4.2. Talent putting the flask in the incubator.

3. Primary Human B-cell Engineering

3.1. Prepare the CRISPR-Cas9 transfecting substrate by mixing 1 microliter of chemically modified sgRNA with 1.5 microliters of chemically modified *Streptococcus pyogenes* Cas 9 nuclease per transfection reaction **[1]**. For control, add 1 microliter of TE buffer instead of sgRNA into a 0.2-milliliter tube of an 8-tube strip **[2]**.

3.1.1. Talent adding sgRNA and Cas 9 nuclease to a PCR tube.

3.1.2. Talent adding TE buffer to the control tube, with the TE container in the shot.

3.2. ~~**[1]**~~. Turn on an electroporator **[2]** and prepare the nucleofection reagents **[3]**.

3.2.1. ~~Talent transferring transfection substrate to a tube on a strip~~.

3.2.2. Talent turning on the electroporator. Vid NOTE: Also in 3.6.3, talent programs electroporator in both clips.

3.2.3. Talent preparing reagents. *Video Editor: Show Table 2 as an inset.*

3.3. Count and transfer 1 million B cells per transfection reaction into a sterile conical tube **[1]**. Bring the volume to 15 milliliters with sterile PBS **[2]**, and centrifuge at 400 × *g* for 5 minutes **[3]**. ~~**[4]**~~.

3.3.1. Talent transferring cells into a tube

3.3.2. Talent adding PBS to the tube, with the PBS container in the shot.

3.3.3. Talent putting the tube in the centrifuge.

3.3.4. Talent preparing the primary cell transfection reagent. Vid NOTE: This is the same step as 3.2.3, and only done once. Talent wants it at 3.2.3 and not 3.3.4.

3.4. After centrifugation, discard the supernatant **[1]**. Resuspend the cells with 10 milliliters of sterile PBS **[2]** and centrifuge at 400 × *g* for 5 minutes **[3]**, then discard the supernatant completely without disturbing the cell pellet **[4]**. *Videographer: This step is difficult and important!*

3.4.1. Talent discarding the supernatant.

3.4.2. Talent resuspending the cells.

3.4.3. Talent putting the tube in the centrifuge.

3.4.4. Talent discarding the supernatant.

3.5. Add 0.5 micrograms of chemically modified GFP mRNA per 1 million B cells to the cell pellet **[1]**. Resuspend the pellet with 20 microliters of primary cell transfection reagent per 1 million B cells and mix gently by pipetting 5 to 6 times **[2]**. Transfer 20.5 microliters per transfection reaction into the 0.2-milliliter tubes of the 8-tube strip **[3]**.

3.5.1. Talent adding the mRNA to the cells.

3.5.2. Talent resuspending the cells pellet by pipetting.

3.5.3. Talent transferring the reaction to a tube on the strip.

3.6. Pipette up and down once to mix and transfer the entire volume into a transfection cuvette **[1]**. Cap and tap the cuvette on the bench gently to ensure that the liquid covers the bottom of the cuvette **[2]**. Use the human primary B-cell protocol on the electroporator for transfection **[3]**. *Videographer: This step is difficult and important!*

3.6.1. Talent pipetting the reaction and transferring it to a cuvette.

3.6.2. Talent capping and tapping the cuvette.

3.6.3. Talent programming the electroporator.

3.7. Rest the electroporated cells in the cuvette for 15 minutes **[1]**, then transfer 80 microliters of the pre-equilibrated B-cell expansion medium into the transfection reaction in the cuvette **[2]**. Place the cuvette in the tissue culture incubator for 30 minutes **[3]**. *Videographer: This step is important!*

3.7.1. Electroporated cells in a cuvette at RT.

3.7.2. Talent transferring medium into the cuvette.

3.7.3. Talent placing the cuvette in the tissue culture incubator.

3.8. Gently pipette a couple of times to mix **[1]** and transfer the whole volume of the sample from the cuvette to an appropriate well of a 48-well tissue-culture plate containing 1 milliliter of the B-cell expansion medium. The final concentration of the cells should be 1 million cells per milliliter **[2]**. *Videographer: This step is important!*

3.8.1. Talent pipetting the sample and transferring the sample to a plate NOTE: 3.8.1 and 3.8.2 are combined. Vid NOTE: Talent pipettes one sample and transfers it before moving on to the next. So slate 3.8.1 is a wide/medium shot of the action and slate 3.8.2 is a closeup of the action.

3.8.2. Talent repeats step 3.8.1 with other samples

3.9. If performing a gene knock-in experiment, transfer recombinant adeno-associated type 6 viral vector at 500,000 multiplicity of infection into the appropriate well containing electroporated cells **[1]**. Place the plate in a tissue culture incubator at 37 degrees Celsius and 5% carbon dioxide with humidity **[2]**. *Videographer: This step is important!*

3.9.1. Talent transferring rAAV6 into a well.

3.9.2. Talent putting the plate in the incubator and closing the door.

Results

4. Results: CRISPR/Cas9-based Genome Engineering of Primary Human B Cells for Gene Knockout and Knock-in

4.1. In the knockout experiment, the B-cell count showed more than 80% viable cells with a slight reduction in cell recovery in both the control and the CD19 knockout samples at 24 hours post-electroporation **[1]**.

4.1.1. LAB MEDIA: Figure 2 A.

4.2. B cells were collected on day 5 post-transfection for flow cytometry and TIDE *(pronounce 'tide')* analyses **[1]**. Representative scatter plots of the control and knockout sample showed 14 and 95% CD19-negative cells, respectively **[2]**, demonstrating a significant reduction in CD19 expression in the knockout samples **[3]**.

4.2.1. LAB MEDIA: Figure 2 B.

4.2.2. LAB MEDIA: Figure 2 B. *Video Editor: Emphasize the CD19- populations in the control and KO plots.*

4.2.3. LAB MEDIA: Figure 2 C.

4.3. Chromatograms of genomic sequencing **[1]** showed double peaks in the CD19 knockout B cells, indicating insertions or deletions of nucleotides post-CRISPR-Cas9-mediated double-stranded breaks **[2]**.

4.3.1. LAB MEDIA: Figure 3 A.

4.3.2. LAB MEDIA: Figure 3 A. *Video Editor: Emphasize the CD19KO chromatogram.*

4.4. Indel analysis of the chromatographs of the knockout samples showed a high percent of indel formation at the CD19 locus, which is consistent with percent CD19 protein loss detected by flow cytometry **[1]**.

4.4.1. LAB MEDIA: Figure 3 B.

4.5. B cells from the knock-in experiment were collected on day 12 post-engineering. Scatter plots showed 64% of EGFP-positive cells in the sample that received the recombinant adeno-associated type 6 viral vector together with RNP **[1]**, whereas none or minimal EGFP-positivity was observed in the control and vector-only samples, respectively **[2]**.

4.5.1. LAB MEDIA: Figure 5 A. *Video Editor: Emphasize the GFP+ population in the KI sample.*

4.5.2. LAB MEDIA: Figure 5 A. *Video Editor: Emphasize the GFP+ population in the control and Vector only.*

4.6. A junction PCR amplification showed 1.5 kilo-base pair amplicons in the knock-in sample **[1]**, and no PCR product was observed in either the control or vector-only sample **[2]**.

4.6.1. LAB MEDIA: Figure 5 B. *Video Editor: Emphasize the KI lane in the gel.*

4.6.2. LAB MEDIA: Figure 5 B. *Video Editor: Emphasize the control and Vector only sample lanes in the gel.*

4.7. Cell counts showed that the engineering process affects cell recovery in the knock-in sample more than the control or the vector-only samples **[1]**. However, all samples quickly rebounded within 3 days after engineering **[2]**.

4.7.1. LAB MEDIA: Figure 5 C. *Video Editor: Emphasize the KI bars.*

4.7.2. LAB MEDIA: Figure 5 C. *Video Editor: Emphasize all day 3 bars.*

Conclusion

5. Conclusion Interview Statements

5.1. **Matthew J. Johnson:** This procedure can also be used to introduce base-editor reagents to alter single-base pairs in order to induce or correct single-point mutations in the B-cell genome and to induce premature stop codons or to alter splice sites, allowing for multiple gene knockouts without the risk of chromosomal transpositions.

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

5.2. **Matthew J. Johnson:** Before this technique was developed, engineering human B cells would require the use of other, more challenging gene engineering approaches. This technique is an easier, less expensive, and quicker alternative. It could potentially be used to synthesize clinical cell therapy products.

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