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Title: Generation of a *RIP1* Knockout U937 Cell Line Using the CRISPR-Cas9 System

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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? **No**

Current Protocol Length
Number of Steps: 18
Number of Shots: 44

Introduction

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

1.1. **Tim LaRocca:** Our research focuses on mammalian programmed cell death, specifically studying the balance between apoptosis and necroptosis in the context of hyperglycemia.

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

What are the most recent developments in your field of research?

1.2. **Tim LaRocca:** Most recently, the cell death protein RIP1 has been shown to modulate gene expression during necroptosis.

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

What are the current experimental challenges?

1.3. **Tim LaRocca:** Our current experimental challenge is genetically manipulating immune cells that grow in suspension, particularly knocking out the gene for RIP1 using CRISPR-Cas9.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.3.1*

What advantage does your protocol offer compared to other techniques?

1.4. **Tim LaRocca:** Our protocol offers 2 distinct advantages. It improves transfection methods for immune cells that grow in suspension by using lentivirus, and it eliminates the need for single cell cloning during the process of creating a genetic knockout using CRISPR-Cas9.

1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 3.4.1*

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

1.5. **Tim LaRocca:** Our results pave the way to study the role of RIP1 in the hyperglycemic shift to necroptosis.

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):

What motivated you to choose JoVE for publishing your research?

1.6. **Tim LaRocca:** We have published with Jove twice previously and had a great experience.
   1.6.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

How does the research community benefit from video publications as compared to standard text publications?

1.7. **Tim LaRocca:** Biology is a very visual discipline. By that nature, many students in this discipline learn by direct observation. A video publication allows for this observation while text publications do not.
   1.7.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

Protocol

2. Harvesting *RIP1*-Targeting CRISPR gRNA Lentiviral Vector with Cas9 Endonuclease and Puromycin Resistance from *Escherichia coli*

Demonstrator: Matthew Deragon

Protocol

2.1. To begin, quadrant streak *Escherichia coli (Esh-uh-REE-kee-uh KOH-lie)* harboring the lentiviral vector guide RNA *(R-N-A)* onto Luria Bertani or *(L-B)* agar plates supplemented with 100 micrograms per milliliter of Ampicillin **[1]**.

2.1.1. WIDE: Talent quadrant-streaking *E. coli* onto LB agar plates with a sterile loop.

2.2. Incubate the plates at 37 degrees Celsius for 1 to 2 days **[1]** until individual colonies are visible in the most dilute area on the plate **[2]**.

2.2.1. Talent placing the plates into the incubator.

2.2.2. ECU: Shot of individual colonies from the plate.

2.3. After incubation, using a sterile loop, select a single colony and add it to 5 milliliters of LB broth supplemented with 100 micrograms per milliliter of Ampicillin in a 50-milliliter conical tube **[1]**. Vent and tape down the cap of the tube **[2]**. Incubate the tube in an orbital shaker at 37 degrees Celsius and 225 rpm *(r-p-m)* for 8 hours **[3]**.

2.3.1. Talent picking a single colony from the plate using a sterile loop and transferring it to a broth containing a 50 mL tube.

2.3.2. Talent venting the tube cap and securing it with tape.

2.3.3. Talent placing the tube into an orbital shaker.

2.4. Meanwhile, add 40 milliliters of LB broth supplemented with 100 micrograms per milliliter of Ampicillin into four 50-milliliter tubes **[1]**.

2.4.1. Talent pipetting LB broth with Ampicillin into the four 50-milliliter conical tubes.

2.5. After incubation, transfer 40 microliters of the grown culture into each of the four tubes **[1]**. Vent and tape down the caps of the tubes **[2]** before incubating them in an orbital shaker at 37 degrees Celsius and 225 rpm *(r-p-m)* for 12 to 16 hours **[3]**.

2.5.1. Talent transferring 40 microliters of the grown culture into the 50 mL tube.

2.5.2. Talent venting the tube caps and securing it with tape.

2.5.3. Talent placing the tubes into an orbital shaker.

2.6. The next day, centrifuge the cultures at 3220 *g* in a swinging bucket rotor for 20 minutes **[1]**. Decant the supernatants into a waste beaker **[2]** and combine all four pellets into 10 milliliters of LB broth supplemented with Ampicillin **[3]**.

2.6.1. Talent placing the tubes in a centrifuge.

2.6.2. Talent decanting supernatants into a labeled waste beaker.

2.6.3. Talent resuspending the combined pellets in LB broth with Ampicillin.

2.7. Centrifuge the combined pellets at maximum speed in a swinging bucket rotor for 20 minutes to obtain a single pellet **[1]** and discard as much of the supernatants as possible into the waste beaker **[2]**.

2.7.1. Talent placing the resuspended pellet into the centrifuge for the second spin.

2.7.2. Talent decanting the remaining supernatants into the waste beaker.

3. Transfection of HEK293T Cells with *RIP1*-Targeting CRISPR gRNA Lentiviral Vector

3.1. To begin, seed HEK293T *(H-E-K-2-9-3-T)* cells at a concentration of 3×10^6 to 5×10^6 cells into a 10-centimeter plate containing DMEM *(D-M-E-M)* **[1-TXT]**. Incubate the plate overnight at 37 degrees Celsius with 5% carbon dioxide **[2]**.

3.1.1. WIDE: Talent pipetting HEK293T cells into a 10-centimeter plate containing the DMEM medium. **TXT: DMEM supplemented with 4.5 g/L D-glucose, L-glutamine, 110 mg/L sodium pyruvate, and 10 % heat-inactivated FBS**

3.1.2. Talent placing the plate into the incubator.

3.2. Meanwhile, prepare the transfection mixture using a 1:1:1:1 *(One-to-one-to-one-to-one)* ratio of plasmids and a 3:1 *(three-to-one)* ratio of transfection reagent to plasmid DNA with reduced serum medium **[1-TXT]**.

3.2.1. Talent collecting the plasmids and transfection reagents and setting up the ratios on a working platform. **TXT: Experimental CRISPR plasmid: pLP1: pLP2: pLP/VSVG:1:1:1:1** *Video Editor: This is ratio*

3.3. Mix 75 microliters of transfection reagent, 2500 microliters of reduced serum medium, and 25 micrograms of total plasmid DNA *(D-N-A)* **[1]**. Allow the mixture to incubate at room temperature for 15 to 30 minutes **[2]**.

3.3.1. Talent measuring and combining the transfection reagent, reduced serum medium, and plasmid DNA into a sterile tube.

3.3.2. Shot of the prepared transfection mixture incubating on the benchtop.

3.4. Next, carefully pipette the transfection mixture onto the confluent HEK293T cells in the plate containing media **[1]**. Gently swirl the plate to ensure adequate homogenization of the transfection mix and media **[2].** Incubate the plate at 37 degrees Celsius for 48 hours **[3]**.

3.4.1. Talent pipetting the transfection mixture into the cell culture plate.

3.4.2. Talent swirling the plate to mix the transfection mixture with the media.

3.4.3. Talent placing the plate into the incubator.

4. Transduction of U937 Cells with Lentivirus Containing Supernatant

4.1. After 2 days of incubation, transfer the media from the producer HEK293T cell plate into a 15-milliliter conical tube **[1]**. Centrifuge the tube at 800 *g* for 5 minutes to pellet any remaining HEK293T cells **[2]**. Carefully remove all the virus-containing supernatant without disturbing the pellet **[3]** and transfer it to a new 15-milliliter conical tube **[4]**.

4.1.1. Talent transferring the media from the HEK293T cell plate into a 15-milliliter conical tube.

4.1.2. Talent placing the tube into the centrifuge.

4.1.3. Talent carefully removing the supernatant without disturbing the pellet.

4.1.4. Talent transferring the virus-containing supernatant into a fresh 15-milliliter conical tube.

4.2. Count and calculate the required volume of the U937 *(U-nine-three-seven)* cell suspension to obtain a pellet of 2×10^6 cells **[1]**. Then, centrifuge the cell suspension in a 15-milliliter tube at 400 *g* for 10 minutes **[2]** and decant the supernatant, leaving the cell pellet in the tube **[3]**.

4.2.1. Talent loading the cell suspension on hemacytometer.

4.2.2. Talent placing the tube in a centrifuge.

4.2.3. Talent decanting the supernatant, leaving the cell pellet in the tube.

4.3. Resuspend the cell pellet in virus-containing supernatant **[1]** and centrifuge the tube at 290 *g* for 60 minutes **[2]**. Following centrifugation, use a pipette to resuspend the pellet with the virus-containing supernatant in the tube **[3]**.

4.3.1. Talent resuspending the cell pellet with virus-containing supernatant.

4.3.2. Talent placing the tube in the centrifuge.

4.3.3. Talent resuspending the pellet in the tube using a pipette.

4.4. Place the tube on an end-over-end rotator for 60 minutes **[1]**. Then, centrifuge the tube at 400 *g* for 10 minutes **[2]** and resuspend the pellet in a 1:1 ratio of the virus-containing supernatants and complete RPMI-1640 *(R-P-M-eye-sixteen-forty)* media supplemented with 10% heat-inactivated FBS *(F-B-S)* **[3]**.

4.4.1. Talent placing the tube on an end-over-end rotator.

4.4.2. Talent transferring the tube in a centrifuge.

4.4.3. Talent resuspending the pellet in the virus-containing and RPMI-1640 media mixture.

4.5. Transfer the cell mixture to a 10-centimeter tissue culture plate **[1]** and incubate the plate at 37 degrees Celsius for 48 hours **[2]**.

4.5.1. Talent transferring the cell mixture to a tissue culture plate.

4.5.2. Talent placing the plate in the incubator.

4.6. After incubation, centrifuge the U937 cells at 400 *g* for 10 minutes **[1]** and remove the supernatant **[2]**. Resuspend the pellet with complete RPMI-1640 media supplemented with 10% heat-inactivated FBS and 5 micrograms per milliliter of puromycin **[3]**. Transfer the cell culture to a T25 *(T-twenty-five)* flask **[4]**.

4.6.1. Talent placing the tube in a centrifuge.

4.6.2. Talent removing supernatant from the tube.

4.6.3. Talent resuspending the pellet in RPMI-1640 medium.

4.6.4. Talent transferring the resuspended cell culture to a T25 flask.

4.7. Incubate the cells at 37 degrees Celsius for 2 to 3 weeks **[1]**, checking the cells every 1 to 2 days for signs of growth **[2]**.

4.7.1. Talent placing the flask in the incubator.

4.7.2. Talent visually inspecting the flask for growth progress.

Results

5. Representative Results

5.1. Western blot analysis confirmed the loss of RIP1 *(Rip-one)* protein expression in RIP1 CRISPR *(kris-per)* mutant cells, with no detectable signal compared to the strong signal in wild-type and non-targeting control cells **[1]**.

5.1.1. LAB MEDIA: Figure 2 *Video editor: Highlight the top blot labeled "RIP1" and compare the absence of the black band in the KO lane to the strong black bands in the WT and NTC lanes*

5.2. Treatment with TCZ *(T-C-Z)* induced significant necroptotic cell death in wild-type and non-targeting control cells **[1-TXT]**, which was reversed by necrostatin-1s treatment **[2]**.

5.2.1. LAB MEDIA: Figure 3 **TXT: TCZ: TNF-α, cycloheximide, and zVAD-fmk** *Video editor: Highlight the black bars in the WT and NTC groups.*

5.2.2. LAB MEDIA: Figure 3 *Video editor: Highlight the white bars in the WT and NTC groups.*

5.3. In contrast, RIP1 CRISPR knockout cells showed significantly lower cell death compared to wild-type and non-targeting control cells **[1]**, with no effect of necrostatin-1s, indicating a loss of RIP1 function **[2]**.

5.3.1. LAB MEDIA: Figure 3 *Video editor: Highlight the black bar in the KO group and then in the WT and NTC groups.*

5.3.2. LAB MEDIA: Figure 3 *Video editor: Highlight the white bars and "ns" in the KO group.*

5.4. Live cell fluorescence microscopy showed a significant increase in mitochondrial superoxide production in wild-type and non-targeting control cells following TCZ treatment **[1]**, which was reduced upon necrostatin-1s treatment **[2].**

5.4.1. LAB MEDIA: Figure 4A *Video editor: Emphasize the red dots in the WT and NTC images in the Vehicle panel*

5.4.2. LAB MEDIA: Figure 4A *Video editor: Emphasize WT and NTC images in the Nec-1s panel*

5.5. RIP1 CRISPR knockout cells exhibited minimal mitochondrial superoxide production, with no observable effect of necrostatin-1s treatment **[1].**

5.5.1. LAB MEDIA: Figure 4A *Video editor: Emphasize KO images in the Vehicle and Nec-1s panels*

Pronunciation Guide:

1. *Escherichia coli*

- **Pronunciation link:** https://www.merriam-webster.com/dictionary/E.%20coli
- **IPA:** /ˌɛʃəˈrɪkiə ˈkoʊlaɪ/
- **Phonetic Spelling:** esh-uh-RIK-ee-uh KOH-liemerriam-webster.com+7merriam-webster.com+7merriam-webster.com+7

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2. CRISPR

- **Pronunciation link:** https://www.merriam-webster.com/dictionary/CRISPR
- **IPA:** /ˈkrɪspər/
- **Phonetic Spelling:** KRIS-permerriam-webster.com+10merriam-webster.com+10merriam-webster.com+10

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3. Puromycin

- **Pronunciation link:** https://www.merriam-webster.com/dictionary/puromycin
- **IPA:** /ˌpjʊrəˈmaɪsɪn/
- **Phonetic Spelling:** pyoo-roh-MY-sinmerriam-webster.com+5merriam-webster.com+5merriam-webster.com+5

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4. Apoptosis

- **Pronunciation link:** https://www.merriam-webster.com/dictionary/apoptosis
- **IPA:** /ˌæpəpˈtoʊsɪs/
- **Phonetic Spelling:** ap-uhp-TOH-sis

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5. Hyperglycemia

- **Pronunciation link:** https://www.merriam-webster.com/dictionary/hyperglycemia
- **IPA:** /ˌhaɪpərglaɪˈsiːmiə/

- **Phonetic Spelling:** hy-per-gly-SEE-mee-uhmerriam-webster.com+18merriam-webster.com+18merriam-webster.com+18

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6. Necroptosis

- **Pronunciation link:** https://www.merriam-webster.com/dictionary/necroptosis
- **IPA:** /ˌnɛkrəpˈtoʊsɪs/
- **Phonetic Spelling:** nek-rop-TOH-sis

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7. Cycloheximide

- **Pronunciation link:** https://www.howtopronounce.com/cycloheximide
- **IPA:** /ˌsaɪkloʊˈhɛksɪmaɪd/
- **Phonetic Spelling:** sy-kloh-HEK-sih-mide

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8. zVAD-fmk

- **Pronunciation link:** No confirmed link found
- **IPA:** /ziː-væd-ɛf-ɛm-keɪ/
- **Phonetic Spelling:** zee-VAD-F-M-K

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9. Necrostatin-1s

- **Pronunciation link:** No confirmed link found
- **IPA:** /ˌnɛkroʊˈstætɪn wʌn ɛs/
- **Phonetic Spelling:** nek-roh-STAT-in one Smerriam-webster.com

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10. RPMI-1640

- **Pronunciation link:** No confirmed link found
- **IPA:** /ˌɑːrpiːɛmˈaɪ sɪkstin ˈfɔːrti/
- **Phonetic Spelling:** R-P-M-eye sixteen-fortymerriam-webster.com+5merriam-webster.com+5merriam-webster.com+5

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11. HEK293T

- **Pronunciation link:** No confirmed link found

- **IPA:** /ˌeɪtʃiːˈkiː tuː naɪn θriː tiː/
- **Phonetic Spelling:** H-E-K two-nine-three T

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12. U937

- **Pronunciation link:** No confirmed link found
- **IPA:** /juː naɪn θriː sɛvən/
- **Phonetic Spelling:** U-nine-three-seven