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Title: A new toolkit for evaluating gene functions using conditional Cas9 stabilization

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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. **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? **Possibly.**

   If **Yes**, how far apart are the locations?

   Near-by buildings all walking distance (2 - 10 meters)

Current Protocol Length

Number of Steps: 16
Number of Shots: 52

Introduction

1. Introductory Interview Statements

1.1. **Polona Šafarič Tepeš:** This method provides fast temporally controlled CRISPR/Cas9 gene editing under Shield-1, offering less off-target effect, lower cell toxicity and higher internally-controlled gene editing compared to constitutive activation of Cas9.

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

1.2. **Polona Šafarič Tepeš:** The main advantage of this protocol is that it can be used for characterization of essential genes as well as tumor survival genes. The stabilization of DD-Cas9 is independent of its expression, allowing the gene of interest to be co-expressed under the same promoter.

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

1.3. **Polona Šafarič Tepeš:** This method can be easily applied in in-vivo and in-vitro systems. Shield-1 can also penetrate through the blood-brain barrier, making it easy to study genes involved in brain development.

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

Protocol

2. Lentiviral preparation

2.1. Evenly seed healthy HEK293T *(spell out)* at a density of 1 to 2 million cells per 10-centimeter tissue culture plate by using 10 milliliters of Glucose DMEM with 10% filtered FBS **[1-TXT]**. Incubate the cells in the tissue culture incubator, at 5% carbon dioxide and 37 degrees Celsius for 20 hours **[2]**.

2.1.1. Talent seeding healthy HEK293T cells in tissue culture plate. **TEXT: HEK293T-Human Embryo kidney 293 cell line**

2.1.2. Talent incubating the plate.

2.2. On the next day, confirm that the cells reached 70% confluency by brightfield microscopy and that they are evenly dispersed across the plate **[1]**. Change media 1 hour before the transfection with a final volume of 10 milliliters **[2]**.

2.2.1. Talent observing the plate under microscope.

2.2.2. Talent changing the media of the cells in the plate.

2.3. Prepare 2 tubes with 500 microliters of warm media **[1]**. Add 25 microliters of transfection reagent to tube 1 **[2]** and incubate at room temperature for 5 minutes **[3]**.

2.3.1. Talent adding the mixture in two tubes containing warm media.

2.3.2. Talent adding transfection reagent in one tube.

2.3.3. Talent incubating the tube at room temperature.

2.4. To the other tube, add a mixture of plasmids **[1-TXT]**. Mix tube 1 with tube 2 to form a transfection mixture **[2]** and incubate at room temperature for 20 minutes **[3]**.

2.4.1. Talent adding mixture of plasmids in another tube. **TEXT: Refer to Text Manuscript for plasmid mixture**

2.4.2. Talent mixing both the tubes

2.4.3. Talent incubating the transfection mixture tube at room temperature.

2.5. Add the transfection mixture dropwise to the HEK293T cells **[1]** and incubate the plate in the tissue culture incubator **[2]**. After 18 hours, carefully change the media to 10

milliliters of fresh DMEM with 10% FBS to remove the transfection reagent **[3]**. Incubate the cells for the next 48 hours **[4]**.

2.5.1. Talent adding the transfection mixture to HEK293T cells in the plate.

2.5.2. Talent incubating the plates in the incubator.

2.5.3. Talent adding fresh media into the cells.

2.5.4. Talent incubating the plates in the incubator for 48 hours.

2.6. After 48 hours, collect the supernatant with a 10-milliliter syringe **[1]** and pass it through a 0.45 micrometer filter **[2]**. Aliquot the virus supernatant **[3]** and store it at minus 80 degrees Celsius **[4]**.

2.6.1. Talent collecting the supernatant in syringe.

2.6.2. Talent passing the supernatant through the filter.

2.6.3. Talent aliquoting the solution in the tube.

2.6.4. Talent storing the tube in minus 80 degrees.

3. Determining virus titer and transduction efficacy with flow cytometry

3.1. For determining virus titer, seed 5 million HEK293T cells per well into two 6-well plates **[1-TXT]**. Incubate both plates at 37 degrees Celsius and 5% carbon dioxide overnight to reach 50 to 60% confluency **[2]**. On the next day, use one of the 6-well plates to count the cells in the 6 wells **[3]**.

3.1.1. Talent seeding HEK293T cells in two 6-well plates. **TEXT: Use one plate for counting next day**

3.1.2. Talent incubating both the plates.

3.1.3. Talent counting the cells in one plate.

3.2. Thaw the virus aliquot that will be used to perform the serial dilution **[1]** and add 8 micrograms per milliliter of polybrene reagent **[2]**. Prepare DMEM with 10% FBS for serial dilution of the virus and add the polybrene reagent **[3]**.

3.2.1. Talent thawing the virus aliquot.

3.2.2. Talent adding polybrene reagent to the aliquot.

3.2.3. Talent adding polybrene reagent in DMEM media.

3.3. Prepare 2 milliliters of ten-fold serial dilutions of the lentivirus from 0.1 to 0.0001 in polybrene-containing media **[1]**. Remove media from the 6-well plate **[2]** and add 1 milliliter of viral dilutions to the wells, leaving one well with media alone as negative control and one well with 100% virus **[3]**. Incubate the cells for 24 hours **[4]**. *Videographer: This step is difficult!*

3.3.1. Talent preparing serial dilutions of the lentivirus.

3.3.2. Talent removing media from the 6-well plate.

3.3.3. Talent adding prepared viral dilutions to the wells.

3.3.4. Talent incubating the cells.

3.4. On the next day, remove the media with the virus from the 6-well plates **[1]** and change it to 2 milliliters of fresh DMEM with 10% FBS **[2]**. Incubate the cells for 48 to 72 hours **[3]**, observing GFP with a fluorescent microscope every day **[4]**.

3.4.1. Talent removing the media from the plates.

3.4.2. Talent adding fresh media.

3.4.3. Talent incubating the plates again.

3.4.4. Talent observing the plates under microscope.

3.5. After 48 to 72 hours, detach the cells **[1]** and resuspend them in MACS buffer **[2-TXT]**. Use a flow cytometer to determine the percentage of GFP expression and calculate the virus titer using the formula given in the text manuscript **[3]**. *Videographer: This step is difficult!*

3.5.1. Talent detaching the cells. **NOTE: Check if this step is skipped.**

3.5.2. Talent resuspending the detached cells in MACS buffer. **TEXT: MACS-Magnetic-activated cell sorting** **NOTE: Check if this step is skipped.**

3.5.3. Talent determining the GFP expression using flow cytometer.

4. Lentiviral transduction of target cells and conditional induction of Cas9 mediated gene editing

4.1. Plate the cell line of interest in a 10-centimeter plate **[1]** and incubate overnight to reach confluency of 50 to 60% **[2]**. Then, infect the cells with 500 to 2000 microliters of virus particles in a 10-milliliter total volume of culture medium **[3]**. Incubate the viral media for 24 hours **[4]**. *Videographer: This step is important!*

4.1.1. Talent plating the cells on plate.

4.1.2. Talent incubating the plate overnight.

4.1.3. Talent infecting the cells with virus particles in culture medium.

4.1.4. Talent incubating the medium.

4.2. On the next day, change the viral media to culture media **[1]** and determine the percentage of GFP positive cells by using flow cytometry **[2]**. Select GFP positive cells by using B FACS *(B-fax)* Aria II *(two)* for cell sorting **[3]**. *Videographer: This step is important!*

4.2.1. Talent changing the viral media to culture media.

4.2.2. Talent determining the GFP positive cells in flow cytometry.

4.2.3. Talent selecting the GFP positive cells.

4.3. Expand positively selected cells **[1]** and freeze a stock **[2]**. Plate the positively selected cells and untransduced cells in 12-well plates separately 24 hours after infection **[3]**. **NOTE: Change of talent from here.**

4.3.1. Talent expanding the positively selected cells.

4.3.2. Talent freezing stocking the positively selected cells.

4.3.3. Talent plating the cells on 12-well plates.

4.4. Wait until the cells attach and change the media to cell culture media containing 200 nanomolar Shield-1 **[1]**. Replace the media in the plates with transduced and untransduced-cells, leaving 2 wells per plate with regular media as a negative control **[2]**. *Videographer: This step is important!*

4.4.1. Talent changing the media from the plates containing Shield-1.

4.4.2. Talent replacing the media in the plates containing transduced and untransduced cells.

4.5. Incubate and extract proteins from each well at 0, 2, 6, 12, 24, 48, and 72 hours after adding Shield-1 to the wells **[1]**. *Videographer: This step is important!*

4.5.1. Talent incubating the plates.

4.6. Then, remove the media with Shield-1 from the rest of the wells **[1]** and change it for regular cell media **[2]**. Collect the proteins from those wells at 2, 6, and 12 hours after changing the media **[3]**. *Videographer: This step is important!*

4.6.1. Talent removing the media with Shield-1 from the wells.

4.6.2. Talent adding regular medium in the wells.

4.6.3. Talent putting the plate in the incubator.

Results

5. Analysis of reversibility and rapidity of destabilized DD-Cas9 protein

5.1. The induction of Cas9 protein was confirmed by its expression levels being similar amongst the transduced cells and the vehicle, notwithstanding the presence or absence of Shield-1 **[1]**.

5.1.1. LAB MEDIA: Figure 2B.

5.2. There is sufficient induction of Cas9 expression in the transduced A549 cell line 2 hours after treatment with Shield-1 **[1]** which becomes negligible within 6 to 12 hours after withdrawal of Shield-1 **[2]**.

5.2.1. LAB MEDIA: Figure 3. *Video editor show the gradual increase in the intensity of the α-Flag band after 2 hours.*

5.2.2. LAB MEDIA: Figure 3. *Video editor show the gradual decrease in the intensity of the α-Flag band 2 hours after withdrawal of Shield-1.*

5.3. Shield-1 treated transduced A549 cells decreased in number after 48 hours without affecting the Renilla sample **[1]**, which was validated by immunoblotting using an antibody against the depleted RPA3 protein **[2]**. Gene editing inversion or indel mutations were confirmed using Surveyor nuclease assays **[3]**.

5.3.1. LAB MEDIA: Figure 4A.

5.3.2. LAB MEDIA: Figure 4B.

5.3.3. LAB MEDIA: Figure 4C.

5.4. The lentivirus vector construct designed was a bicistronic system bearing another gene of interest under the same EF-1a promoter **[1]**. A modified fluorescent protein, P2A was added to trace infected cells **[2]**.

5.4.1. LAB MEDIA: Figure 5A

5.4.2. LAB MEDIA: Figure 5A.

5.5. The expression of the mVenus protein was observed in the vehicle and A549 transduced cells independent of DD-Cas9 expression and Shield-1 treatment **[1]**.

5.5.1. LAB MEDIA: Figure 5B.

Conclusion

6. Conclusion Interview Statements

6.1. **Polona Šafarič Tepeš:** The efficient transduction of the cell line with the DD-Cas9 vector is one of the rate-limiting steps where having healthy HEK-293T cells and using low passage number is crucial.

6.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll 2.1*

6.2. **Polona Šafarič Tepeš:** Optimization of the ratio between DD-Cas9 plasmid, packaging plasmid, and envelope plasmid is very important for optimal transfection along with the amount of Shield-1 needed to stabilize DD-Cas9 in the target cells.

6.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll 2.3, 4.6*

6.3. **Polona Šafarič Tepeš:** DD-Cas9 with the Cre plasmid can be used to study the genetic interaction *in vivo* systems based on the Cre-lox system.

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