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Title: Epigenetic Engineering of K562 Cells: Dual-Vector Episomal Strategy for Stable Targeted DNA Methylation using dCas9-DNMT3A and -HDAC1 Fusion Proteins

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Author Questionnaire

1. We have marked your project as author-provided footage, meaning you film the video yourself and provide JoVE with the footage to edit. JoVE will not send the videographer. Please confirm that this is correct.

√ Correct

2. Microscopy: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **No**

3. Software: Does the part of your protocol being filmed include step-by-step descriptions of software usage? **No**

4. Proposed filming date: To help JoVE process and publish your video in a timely manner, please indicate the proposed date that your group will film here: **11/14/2025**

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Current Protocol Length

Number of Steps: 25
Number of Shots: 55

Introduction

INTRODUCTION:

~~What is the scope of your research? What questions are you trying to answer?~~

1.1. **Irene Dereki:** Our research aims to develop a stable, non-integrative system for targeted DNA methylation, exploring durable epigenetic editing in K562 cells.

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

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

1.2. **Irene Dereki:** Recent advances in the field include dual-effector CRISPR/dCas9 systems combining epigenetic enzymes for synergistic, stable, and locus-specific epigenetic modification.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. ~~*Suggested B-roll: 2.5.1*~~

CONCLUSION:

~~What research gap are you addressing with your protocol?~~

1.3. **Irene Dereki:** We address the lack of non-integrative, long-term delivery systems enabling precise, stable epigenetic modifications without altering the host genome.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. ~~*Suggested B-roll: 3.3.1*~~

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

1.4. **Irene Dereki:** Our findings establish a reliable tool for studying gene regulation via targeted methylation and support developing possible precise epigenetic therapies.

1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. ~~*Suggested B-roll: 4.2.1*~~

~~What questions will future research focus on?~~

1.5. **Irene Dereki:** Future research will explore improving episome delivery, enhancing methylation efficiency, minimizing off-target effects, and adapting the system for primary or in vivo models.

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

Protocol

2. DNA Preparation for Transfection

Demonstrator: Irene Dereki

2.1. To begin, maintain K562 cells in T-75 culture flasks at 37 degrees Celsius with 5 percent carbon dioxide and humidified conditions **[1-TXT]**.

2.1.1. **WIDE:** Talent placing T-75 flasks into a cell culture incubator set to 37 degrees Celsius with 5 percent carbon dioxide. **TXT: Use DMEM supplemented with 10% FBS and 1× Penicillin-Streptomycin**

2.2. Quantify the episomal DNA to be used for transfection using a spectrophotometer at 260 nanometers **[1].** Use 8 micrograms of total plasmid DNA per transfection by preparing 4 micrograms of each plasmid for a co-transfection **[2]** and mix the required amount with 90 microliters of ultra-pure water **[3]**.

2.2.1. Talent placing the sample in spectrophotometer.

2.2.2. LAB MEDIA: Figure 1.

2.2.3. Talent inverting the tube to mix.

2.3. Now, add 10 microliters of 3 molar sodium acetate solution and 2.5 volumes of 100 percent ethanol to the diluted DNA solution **[1]**. Mix the DNA solution thoroughly **[2]** and incubate it overnight at minus 20 degrees Celsius to allow DNA precipitation **[3]**.

2.3.1. Talent adding sodium acetate followed by ethanol to the DNA-containing microcentrifuge tube.

2.3.2. Talent closing the tube cap, vortexing briefly.

2.3.3. Talent placing the tube into a minus 20 degree Celsius freezer.

2.4. On the following day, centrifuge the DNA precipitation tube at 11,000 *g* for 15 minutes at 4 degrees Celsius **[1]**.

2.4.1. Talent placing tube into a centrifuge and setting it to 11,000 g at 4 degrees Celsius for 15 minutes.

2.5. Under sterile conditions in a biosafety cabinet, carefully discard the supernatant from the tube **[1]**. Add 500 microliters of 70 percent ethanol to the tube to wash the pellet **[2]** and centrifuge again at 11,000 *g* for 5 minutes at 4 degrees Celsius **[3]**.

2.5.1. Talent inside a biosafety cabinet gently pouring off the supernatant from the DNA pellet.

2.5.2. Talent adding 70 percent ethanol to the pellet-containing tube.

2.5.3. Talent placing tube in the centrifuge for a 5-minute spin at 11,000 g and 4 degrees Celsius.

2.6. Next, remove the ethanol supernatant completely from the tube **[1]** and let the DNA pellet air-dry until no visible ethanol remains **[2]**.

2.6.1. Talent using a pipette to remove all ethanol from the tube.

2.6.2. Close-up of the tube left open inside the biosafety cabinet.

2.7. Then, resuspend the dried DNA pellet thoroughly in 50 microliters of DMEM **[1].** Keep 400 nanograms of the resuspended DNA, approximately 2.5 microliters, in a new tube **[2]**, and store the plasmid-DMEM solution at 4 degrees Celsius until further use **[3]**.

2.7.1. Talent pipetting DMEM into the tube and mixing to resuspend the DNA pellet.

2.7.2. Talent pipetting 2.5 microliters of the DNA-DMEM mix in a new tube.

2.7.3. Talent placing the DNA-DMEM tube in a 4 degree Celsius refrigerator.

2.8. Run the remaining DNA sample, on a 1 percent agarose gel to verify DNA purification and integrity **[1]**.

2.8.1. Talent pipetting the DNA sample into wells of a 1 percent agarose gel.

3. Preparation, Transfection and Culturing Cells

Demonstrator: Irene Dereki

3.1. Harvest K562 cells from a T-75 flask when cultures reach 50 to 60 percent confluency to ensure optimal transfection efficiency **[1]**.

3.1.1. Talent removing a T-75 flask from incubator.

3.2. Count the harvested cells using a hemocytometer **[1]** and transfer a volume containing 2 × 10^5 cells into a 35-millimeter culture dish containing 900 microliters of DMEM without FBS or antibiotics **[2]**.

3.2.1. Talent viewing the sample under a microscope.

3.2.2. Talent pipetting the calculated cell volume into a 35-millimeter dish containing serum-free DMEM.

3.3. Place the culture dish in a 37 degrees Celsius incubator with 5 percent carbon dioxide to maintain the cells in a ready state for transfection **[1]**. Use a Lipofectamine-based transfection kit to deliver plasmids, following the manufacturer's instructions and adjusting volumes if needed **[2]**.

3.3.1. Talent placing the culture dish into a humidified incubator.

3.3.2. Talent holding the transfection kit and reading the manufacturer's instruction leaflet at the bench.

3.4. Add 3 microliters of P3000 Reagent to the previously prepared plasmid-DMEM solution **[1]**. Mix gently and incubate the mixture at room temperature for 5 minutes **[2]**.

3.4.1. Talent pipetting 3 microliters of P3000 reagent into the DNA solution tube.

3.4.2. Talent gently flicking or pipetting the tube to mix and placing it on the benchtop for incubation.

3.5. Now, add 5 microliters of Lipofectamine 3000 Reagent along with enough DMEM to reach a final volume of 100 microliters **[1]**. Incubate this transfection mix at room temperature for 15 minutes **[2]**.

3.5.1. Talent adding Lipofectamine 3000 and adjusting with DMEM to 100 microliters in a clean microcentrifuge tube.

3.5.2. Talent placing the tube on the benchtop and starting a 15-minute timer.

3.6. Add the full 100 microliters of the transfection mixture to the 35-millimeter culture dish containing the cells **[1]**. Gently shake the dish to disperse the mixture evenly **[2]**, then return it to the incubator **[3]**.

3.6.1. Talent pipetting the transfection mix onto the cultured cells in the 35-millimeter dish.

3.6.2. Talent gently shaking the dish in a circular motion.

3.6.3. Talent placing the dish back inside the incubator.

3.7. At 24 hours post-transfection, add 3 milliliters of full medium—DMEM supplemented with 10 percent FBS and 1× penicillin-streptomycin to each dish to promote proliferation of transfected cells **[1]**. At 72 hours post-transfection, begin selection by adding G-418 antibiotic to reach a final concentration of 1 milligram per milliliter **[2]**.

3.7.1. Talent adding 3 milliliters of complete culture medium.

3.7.2. Talent adding G-418 solution to the dish and swirling gently to mix.

4. Isolation of Single Clones and Extraction of Nucleic Acids

Demonstrator: Irene Dereki

4.1. Once the selection process is established and total cell count exceeds 1×10^6 **[1]**, harvest the cells by centrifugation at 300 *g* for 6 minutes at 25 degrees Celsius **[2]**. Wash the pellet once with 1× sterile PBS, pH approximately 7.4, and centrifuge again under the same conditions **[3]**.

4.1.1. Talent loading the cell suspension into centrifuge tubes.

4.1.2. Talent placing the sample in a centrifuge.

4.1.3. Talent adding PBS to the pellet, mixing briefly.

4.2. After resuspending the final cell pellet in 1× PBS, determine the cell concentration using a hemocytometer **[1]**. Dilute the suspension to 1×10^6 cells per milliliter, ensuring the cells are fully resuspended **[2]**, and transfer cells to a flow cytometry tube **[3]**.

4.2.1. Talent resuspending the cell pellet in PBS and loading the sample onto a hemocytometer.

4.2.2. Talent diluting the suspension to the required concentration.

4.2.3. Talent transferring the ample to a cytometry tube.

4.3. Next, set up the fluorescence-activated cell sorter to single-cell mode using a 96-well tissue culture-treated plate as the collection device **[1]**. Adjust the software settings depending on the instrument used **[2]**.

4.3.1. Talent inserting flow cytometry tube to the cell sorter.

4.3.2. Shot of the software interface with adjustments made to enable single-cell

sorting mode.

4.4. Gate the primary population by selecting cells based on forward scatter and side scatter to isolate expected size and granularity **[1]**. Exclude debris and highly granular or dead cells, and use forward scatter area versus height or side scatter area versus height plots to gate singlets **[2]**.

4.4.1. Shot of monitor showing gating window with FSC vs SSC and debris exclusion marked on the plot.

4.4.2. Shot of monitor showing highlighted singlet gating using FSC-A vs FSC-H and SSC-A vs SSC-H dot plots.

4.5. Then, pre-fill each well of the 96-well plate with 100 microliters of full medium containing 1 milligram per milliliter of G-418 **[1]** and warm the plate in a 37 degrees Celsius incubator **[2]**. Perform cell sorting in single-cell mode, keeping the sorting rate below 300 events per second to improve accuracy **[3]**.

4.5.1. Talent pipetting medium with G-418 into each well of the plate.

4.5.2. Talent placing the plate into the incubator.

4.5.3. Talent placing plate into the cell sorter and monitoring cell sorting procedure.

4.6. After sorting, inspect each well under a microscope to confirm the presence of a single cell per well **[1]** and place the plate in a 37 degrees Celsius incubator with 5 percent carbon dioxide **[2]**.

4.6.1. Talent observing the 96-well plate under a microscope.

4.6.2. Talent placing the inspected plate into the incubator.

4.7. Allow the single-cell clones to expand individually in the 96-well plate **[1]**. Once clonal growth is evident, add medium containing reduced G-418 to the wells, to a final concentration of 400 micrograms per milliliter to maintain selection pressure **[2]**. Transfer well-growing clones sequentially to 12-well plates, then to T-25 flasks, and finally to T-75 flasks as needed until each clone reaches approximately 1×10^6 cells **[3]**.

4.7.1. Talent removing the plate from the incubator.

4.7.2. Talent adding medium containing reduced G-418 concentration.

4.7.3. Talent showing a 12-well plate, then showing T-25 and T-75 flasks containing cultures.

4.8. Next, collect double cell pellets at regular intervals **[1]**. Store one pellet at minus 20 degrees Celsius for genomic DNA extraction **[2]**. Homogenize a second pellet in 500 microliters of Trizol Reagent **[3]** and store it at minus 20 degrees Celsius for RNA extraction and complementary DNA synthesis **[4]**.

4.8.1. Talent placing a tube with sample in a centrifuge.

4.8.2. Talent placing one pellet tube into a minus 20 degree Celsius freezer.

4.8.3. Talent adding Trizol to the second pellet containing tube.

4.8.4. Talent placing the tube in the freezer.

4.9. Extract genomic DNA from selected clones using phenol-chloroform extraction, followed by ethanol precipitation **[1]**.

4.9.1. Talent adding phenol-chloroform to a sample tube.

4.10. Fully resuspend the extracted DNA in nuclease-free water or TE buffer **[1]**. Measure the DNA concentration and purity using a spectrophotometer before continuing to downstream steps **[2]**. Finally, extract total RNA from the stored cell pellets using Trizol Reagent followed by isopropanol precipitation **[3-TXT]**.

4.10.1. Talent pipetting TE buffer into the DNA pellet tube and mixing gently to dissolve.

4.10.2. Talent placing the sample in spectrophotometer.

4.10.3. Talent removing the cell pellet with Trizol from the freezer. **TXT: Perform PCR, pyrosequencing and cDNA synthesis using the isolated nucleic acids**

Results

5. Results

5.1. Single cells were sorted into 96-well plates using fluorescence-activated cell sorting to isolate those carrying both constructs **[1]**. PCR analysis of 10 expanded clones confirmed that only some retained both constructs, indicated by bands at 230 base pairs and 205 base pairs **[2]**.

5.1.1. LAB MEDIA: Figure 3C.

5.1.2. LAB MEDIA: Figure 3C. *Video editor: Highlight lanes 3 and 4* .

5.2. Pyrosequencing revealed that the positive clone 2 showed increased methylation at multiple CpG sites near the gRNA1 target region compared to the untransfected control **[1]**.

5.2.1. LAB MEDIA: Figure 3D. *Video editor: Highlight the green bars labeled "Clone C2" in the graph within the box.*

Pronunciation Guide:

- **Epigenetic**
  Pronunciation link: https://www.merriam-webster.com/dictionary/epigenetic
  IPA: /ˌɛpɪdʒəˈnɛtɪk/
  Phonetic Spelling: eh·pih·juh·neh·tik
- **Episomal**
  Pronunciation link: No confirmed link found
  IPA: /ˌɛpɪˈsoʊməl/
  Phonetic Spelling: eh·pih·soh·muhl
- **Methylation**
  Pronunciation link: https://www.merriam-webster.com/dictionary/methylation
  IPA: /ˌmɛθəˈleɪʃən/
  Phonetic Spelling: meth·uh·lay·shun
- **CRISPR**
  Pronunciation link: https://www.merriam-webster.com/dictionary/CRISPR
  IPA: /ˈkrɪspər/
  Phonetic Spelling: kris·per
- **DNMT3A**
  Pronunciation link: No confirmed link found
  IPA: /diː.ɛn.ɛm.tiːˈθriː.eɪ/
  Phonetic Spelling: dee·en·em·tee·three·ay
- **HDAC1**
  Pronunciation link: No confirmed link found
  IPA: /eɪtʃ.dækˈwʌn/
  Phonetic Spelling: aych·dak·wun
- **K562**
  Pronunciation link: No confirmed link found
  IPA: /keɪ.faɪv.sɪkˈstiː.tuː/
  Phonetic Spelling: kay·five·sik·stee·too
- **Nanometers**
  Pronunciation link: https://www.merriam-webster.com/dictionary/nanometer
  IPA: /ˈnænəˌmiːtər/
  Phonetic Spelling: nan·uh·mee·ter
- **Spectrophotometer**

Pronunciation link: https://www.merriam-webster.com/dictionary/spectrophotometer
IPA: /ˌspɛk.troʊ.foʊˈtɑː.mə.tər/
Phonetic Spelling: spek·troh·foh·taa·muh·ter

- Microcentrifuge
  Pronunciation link: No confirmed link found
  IPA: /ˌmaɪ.kroʊˈsɛn.trə.fjuːdʒ/
  Phonetic Spelling: my·kroh·sen·truh·fyooj
- Confluency
  Pronunciation link: No confirmed link found
  IPA: /ˈkɒn.fluː.ən.si/
  Phonetic Spelling: kon·floo·en·see
- Hemocytometer
  Pronunciation link: https://www.merriam-webster.com/dictionary/hemocytometer
  IPA: /ˌhiːmoʊsaɪˈtɒmɪtər/
  Phonetic Spelling: hee·moh·sai·tom·ih·ter
- Lipofectamine
  Pronunciation link: No confirmed link found
  IPA: /ˌlɪp.oʊˈfɛk.təˌmiːn/
  Phonetic Spelling: lip·oh·fek·tuh·meen
- Fluorescence
  Pronunciation link: https://www.merriam-webster.com/dictionary/fluorescence
  IPA: /flɔːˈrɛs.əns/
  Phonetic Spelling: floor·es·ens
- Cytometry
  Pronunciation link: https://www.merriam-webster.com/dictionary/cytometry
  IPA: /saɪˈtɒm.ə.tri/
  Phonetic Spelling: sai·tom·uh·tree
- Pyrosequencing
  Pronunciation link: No confirmed link found
  IPA: /ˌpaɪ.roʊˈsiː.kwən.sɪŋ/
  Phonetic Spelling: pie·roh·see·kwen·sing