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Title: Epigenome-Wide CRISPR-Cas9-Based Knockout Screens on Chemoresistant 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? **NO**

- 3. Filming location:** Will the filming need to take place in multiple locations? **NO**

- 4. Testimonials (optional):** Would you be open to filming two short testimonial statements **live during your JoVE shoot**? These will **not appear in your JoVE video** but may be used in JoVE's promotional materials. **NO**

Current Protocol Length

Number of Steps: 34

Number of Shots: 59

Interviews

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

Authors: Please note that **the questions will not appear on screen**. Please answer in **stand-alone full sentence with sufficient context**

INTRODUCTION:

~~**REQUIRED:** Summarize your research focus and the main questions it aims to answer.~~

- 1.1. **Tugba Bagci-Onder:** We investigate epigenetic mechanisms driving chemotherapy resistance in triple-negative breast cancer using CRISPR-based screening approaches.
 - 1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera *Suggested B roll: Figure 1*

~~Describe the research gap or limitation in existing methods and how this protocol addresses them.~~

- 1.2. **Ozlem Yedier-Bayram:** Existing methods fail in chemoresistant cells with multidrug resistance; this protocol optimizes viral delivery and selection for efficient stable transduction.
 - 1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

CONCLUSION:

~~What does this protocol allow researchers to study or measure?~~

- 1.3. **Elif Ayca Guvener:** This protocol enables researchers to study therapy resistance in cancer models and identify epigenetic regulators of chemotherapy resistance using CRISPR screening platforms.
 - 1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

~~Describe the most important consideration or challenge when performing this protocol.~~

- 1.4. **Ozlem Yedier-Bayram:** The most important consideration when screening chemoresistant cells is maintaining library representation and optimizing transduction and selection conditions.
 - 1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

~~How can future studies build on this work? Describe the questions or research directions that can be investigated next.~~

- 1.5. **Tugba Bagci-Onder:** Future studies can better identify therapeutic vulnerabilities of multidrug-resistant cells and develop combination strategies to overcome chemotherapy resistance.
 - 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.

Protocol

Videographer: Please always record an audio slate while filming each shot mentioned in the shotlist.

2. Generation of Taxol-Resistant Triple-Negative Breast Cancer Cell Lines

Demonstrator: Elif Ayca Guvener

- 2.1. To begin, adjust SUM159PT (*Sum-One-Five-Nine-P-T*) adherent cells to 2×10^3 cells per well in 96-well black clear-bottom plates with Ham's F12 (*F-Twelve*) nutrient mix [1] supplemented with the presented components [2]. Incubate the cells for 16 hours in a cell culture incubator at 37 degrees Celsius and 5% carbon dioxide [3].
 - 2.1.1. WIDE: Talent adjusting the density of SUM159PT adherent cells in 96-well black clear bottom plates with supplemented Ham's F12 nutrient mix. **Videographer:** Please obtain CU shot as well for this shot.
 - 2.1.2. TXT ON A PLAIN BACKGROUND
 - 5% FBS
 - 1% Penicillin-Streptomycin
 - 5 μ L/mL insulin
 - 10 mM HEPES
 - 1 μ L/mL hydrocortisone
 - 2.1.3. Talent placing the 96-well plate in the cell culture incubator.
- 2.2. Next, prepare Taxol stock dissolved in dimethyl sulfoxide or DMSO (*D-M-S-O*) [1]. To treat the cells, prepare serial dilutions of Taxol in culture media ranging from 0.3 nanomolar to 10 micromolar and use DMSO alone as a vehicle control [2].
 - 2.2.1. Talent preparing Taxol stock dissolved in DMSO.
 - 2.2.2. Talent preparing serial dilutions of Taxol in culture media.
- 2.3. Treat the cells with 200 microliters of increasing doses of Taxol as triplicates [1]. Leave the plate in a cell culture incubator for 72 hours at 37 degrees Celsius with 5% carbon dioxide [2].
 - 2.3.1. Talent adding 200 microliters of increasing Taxol doses to the cells in triplicate wells.
 - 2.3.2. Talent placing the plate in the cell culture incubator.
- 2.4. After 72 hours of drug treatment, measure cell viability with a luminescence-based ATP quantification cell viability assay [1]. Using a microplate reader, measure luminescence [2]. Normalize the values to DMSO controls. Then calculate the 10% and 50% inhibitory

concentration values by nonlinear regression using a four-parameter logistic dose-response model in appropriate statistical software [3].

2.4.1. Talent adding detection reagent to the cells.

2.4.2. Talent measuring luminescence using the microplate reader.

2.4.3. SCREEN: 71175_screenshot_1.MOV: 00:12-00:25,00:32-00:36, 00:41-00:42

2.5. To start generating chemoresistant cells, seed 2×10^5 SUM159PT cells per well in 6-well plates [1] and incubate overnight at 37 degrees Celsius with 5% carbon dioxide [2].

2.5.1. Talent seeding SUM159PT cells into 6-well plates.

2.5.2. Talent placing the 6-well plate in the cell culture incubator.

2.6. The next day, treat the cells with 10% or 50% inhibitory concentrations of Taxol [1] in 2 milliliters of culture media for 72 hours [2].

2.6.1. Talent adding Taxol-containing culture media to the cells.

2.6.2. Talent placing the plate in the cell culture incubator.

2.7. If the cells tolerate the current drug concentration, split them into two wells. Keep one as a backup control with fresh medium [1] and double the Taxol concentration in the other well. [3]. NOTE: 2.7 and 2.8 are combined in a single shot

2.7.1. Talent splitting the cells into two wells.

2.7.2. Talent replacing the medium in the second well with medium containing twice the previous Taxol concentration.

2.8. While generating resistant cells, passage the parental cells in medium containing DMSO as a control [1] and adjust the DMSO volume to match the amount present in the corresponding Taxol treatment [2].

2.8.1. Talent passaging parental cells in medium containing DMSO

2.8.2. Talent adjusting the DMSO volume in the control medium to corresponding Taxol treatment.

2.9. Measure cell viability every 3 to 4 weeks until a significant difference in the 50% inhibitory concentration values is observed between the parental and resistant cell lines [2].

2.9.1. Talent places the cells in the spectrophotometer microplate reader.

2.10. After obtaining stable resistant cells, maintain them in the maintenance dose during routine culture to preserve the resistant phenotype [1].

2.10.1. Talent adding stable resistant cells in medium containing the maintenance dose of the drug.

2.11. For characterization of resistant cells, perform RNA sequencing, western blotting, qPCR,

and cell viability assays in the presence of the drug [1]. ~~Re-evaluate the resistant phenotype after several passages to demonstrate that the elevated 50% inhibitory concentration phenotype remains stable over time [5].~~ NOTE: The VO for the deleted shot is removed

2.11.1. Talent preparing resistant cell samples for RNA sequencing.

~~2.11.2. Talent analysing the cells using the microplate reader.~~ NOTE: Not filmed

3. Establishment of Optimized Puromycin Selection for Multidrug-Resistant Cells

3.1. Seed SUM159PT Taxol-resistant and parental cells in triplicate into 96-well black clear bottom plates at 2×10^3 cells per well [1]. Incubate the cells overnight at 37 degrees Celsius with 5% carbon dioxide [2].

3.1.1. WIDE: Talent seeding SUM159PT Taxol-resistant and parental cells into 96-well black clear bottom plates in triplicate.

3.1.2. Talent placing the plate in the cell culture incubator.

3.2. Treat the cells with serial dilutions of verapamil while maintaining a constant concentration of Taxol across all treatment conditions [1].

3.2.1. Talent adding serial dilutions of verapamil to the cells.

3.3. After 72 hours of treatment, assess cell viability with a luminescence-based ATP quantification cell viability assay [1]. Determine the verapamil concentration that does not reduce viability on its own but restores sensitivity in resistant cells [2].

3.3.1. Talent places the cells in the microplate reader.

3.3.2. LAB MEDIA: Figure 3E

3.4. To find the puromycin concentration to be used in combination with verapamil, seed SUM159PT Taxol-resistant cells into a 96-well plate at 2×10^3 cells per well [1]. Incubate the plate for 16 hours at 37 degrees Celsius and 5% carbon dioxide [2].

3.4.1. WIDE: Talent seeding SUM159PT Taxol-resistant cells into a 96-well plate.

3.4.2. Talent placing the plate in the cell culture incubator.

3.5. Prepare serial dilutions of puromycin in medium containing the previously determined verapamil concentration [1]. Treat the cells with the puromycin dilutions accordingly for 72 hours [2].

3.5.1. Talent preparing serial dilutions of puromycin in medium containing the previously determined verapamil concentration.

3.5.2. Talent adding the puromycin-containing media to the cells.

3.6. After performing a cell viability assay, determine the minimum puromycin concentration that eliminates all cells and results in 5% viability or less in the presence

of verapamil [2].

3.6.1. SCREEN: 71175_screenshot_2.MOV: 00:06-00:16, 00:20-00:22

3.7. Then use SUM159PT Taxol-resistant cells to calculate viral titration and multiplicity of infection [1].

3.7.1. Representative shot of talent adding viral media to the cells with all the required reagents in the frame.

4. Generation and/or Validation of a Cas9-Expressing Stable Cell Line

Demonstrator: Ozlem Yedier-Bayram

4.1. Take Cas9 lentivirus produced using the LentiCas9_blast vector [1].

4.1.1. WIDE: Talent placing the vial containing lentivirus on the working area.

4.2. ~~To infect the cells to achieve a multiplicity of infection of 10. Calculate the viral dose based on the desired multiplicity of infection. [1].~~ Add the calculated viral volume, based on the desired multiplicity of infection, directly to the cells in culture medium containing 8 micrograms per milliliter Protamine sulfate [1-TXT]. **NOTE: The VO is edited for the deleted shot**

4.2.1. ~~SCREEN: Show calculation of the virus amount required to achieve a multiplicity of infection of 10. NOTE: NOT filmed~~

4.2.2. Talent adding the calculated virus volume to cells in culture medium containing 8 micrograms per milliliter Protamine sulfate. **TXT: Desired MOI = 10**

4.3. Replace the viral medium with fresh medium 16 hours after infection [1].

4.3.1. Talent replacing the viral medium with fresh medium.

4.4. The next day, apply blasticidin selection by replacing the culture medium with medium containing the previously determined concentration of blasticidin [1]. Continue selection until uninfected cells are eliminated [2].

4.4.1. Talent adding culture medium containing the previously determined concentration of blasticidin.

4.4.2. Talent placing the cells in the incubator.

4.5. Validate Cas9 activity with control single-guide RNAs for essential genes or use GFP as a reporter to assess knockout efficiency. [2].

4.5.1. SCREEN: Images of Cas9 validation results on the computer. **NOTE: Use missing media card here**

5. Epigenome-Wide CRISPR Knockout Lentiviral Library Infection and Screening

5.1. Calculate the number of successfully infected cells required to reach the desired library coverage using the presented equation [1].

5.1.1. TEXT ON A PLAIN BACKGROUND

$$N_{infected} = (\text{number of sgRNAs}) \times (\text{target coverage})$$

~~5.2. Calculate the starting cell number for each replicate to compensate for the fraction of uninfected cells following low multiplicity of infection at 0.3 to 0.4 [1]. Determine the transduction rate as the fraction of infected cells based on the Poisson distribution [2].~~

~~5.2.1. SCREEN: Show calculation of the starting cell number per replicate using the transduction rate. NOTE: SC video is not provided. The information is added as an onscreen text at 5.4~~

~~5.2.2. SCREEN: Show determination of the transduction rate from Poisson distribution.~~

~~5.3. Calculate the required viral volume for each replicate [1].~~

~~5.3.1. SCREEN: Show calculation of the required viral volume per replicate.~~

5.4. Prepare independent infection mixtures for each biological replicate [1]. Infect the cells with the calculated viral volume in 8 milliliters of complete culture media containing 8 micrograms per milliliter Protamine sulfate [2-TXT].

5.4.1. Talent preparing separate infection mixtures for each biological replicate

5.4.2. Talent adding the calculated viral volume to cells in complete culture media containing 8 micrograms per milliliter Protamine sulfate. **TXT: Refer to the text for viral volume calculations**

5.5. After 16 hours, replace the viral media with fresh media [1] and incubate the cells at 37 degrees Celsius with 5% carbon dioxide [2].

5.5.1. WIDE: Talent replacing the viral media with fresh media.

5.5.2. Talent placing the cells in the cell culture incubator.

5.6. Apply puromycin selection in the presence of verapamil at the previously determined concentrations to enrich sgRNA-infected cells in SUM159PT Taxol-resistant cells [1]. Continue selection until all cells in the uninfected control plate are eliminated, typically for approximately 3 days [2].

5.6.1. Talent adding selection medium containing puromycin and verapamil to SUM159PT Taxol-resistant cells.

5.6.2. Talent placing cells in the incubator.

5.7. After completion of puromycin and verapamil selection, trypsinize the cells [1] and collect an initial time point, time zero cell pellet from each biological replicate corresponding to the coverage level decided during transduction [2]. Collect the cell pellets as soon as selection is complete, before any cell loss, because they serve as the reference for the initial library distribution [3].

- 5.7.1. Talent adding trypsin to the selected cells.
- 5.7.2. Talent collecting a time zero cell pellet from each biological replicate.
- 5.7.3. Talent collecting cell pellets immediately after selection is completed.
- 5.8. Split the cells into experimental arms while preserving coverage: one arm as the control with DMSO and the other as the treatment with Taxol [1].
 - 5.8.1. Talent splitting the cell population into separate groups.
- 5.9. Maintain the cultures for 14 to 16 population doublings [1]. Calculate the population doubling level using the specified formula [2].
 - 5.9.1. Talent places the cells in the incubator.
 - 5.9.2. TEXT ON A PLAIN BACKGROUND
$$PDL = PDL_s + 3.322 (\log C_f - \log C_i)$$
- 5.10. At each passage, monitor cell viability and growth kinetics. Maintain adequate cell numbers to avoid bottlenecks [1].
 - 5.10.1. SCOPE: The cell viability and growth kinetics is being checked during routine passaging. NOTE: Use missing media card
- 5.11. Upon reaching the desired population doubling level, collect final-timepoint pellets for each replicate and condition, while maintaining library coverage per pellet [1]. Store the pellets at minus 80 degrees Celsius until downstream genomic DNA extraction [2].
 - 5.11.1. Talent collecting final timepoint cell pellets from each replicate and condition.
 - 5.11.2. Talent placing the collected pellets into storage at minus 80 degrees Celsius.

Video editor: Use title card to show the information given below

Perform the following:

- Genomic DNA extraction
- EPIKOL sequencing library preparation from genomic DNA
- Sequencing

Results

6. Results

- 6.1. Resistant cells continued to proliferate despite Taxol treatment [1], while parental cells failed to survive under the same treatment [2].
 - 6.1.1. LAB MEDIA: Figure 2C,D. *Highlight the Taxol-Res sample in the Taxol-treated condition showing visible colonies and the purple bars*
 - 6.1.2. LAB MEDIA: Figure 2C,D. *Video editor: Highlight the Parental sample in the Taxol-treated condition showing the absence of visible colonies and the grey bars*
- 6.2. RNA sequencing identified ABCB1 (A-B-C-B-One) as one of the most upregulated genes in resistant cells [1], followed by other ABC transporters including ABCB4 (A-B-C-B-Four) [2] and ABCA5 (A-B-C-B-Five) [3].
 - 6.2.1. LAB MEDIA: Figure 2E. *Video editor: Highlight the label "ABCB1" on the plot.*
 - 6.2.2. LAB MEDIA: Figure 2E. *Video editor: Highlight the label "ABCB4" on the plot.*
 - 6.2.3. LAB MEDIA: Figure 2E. *Video editor: Highlight the label "ABCA5" on the plot.*
- 6.3. The upregulation of ABCB1 expression in resistant cells was validated by quantitative PCR [1].
 - 6.3.1. LAB MEDIA: Figure 2F. *Video editor: Highlight the Taxol-Res bar, which is visibly higher than the Parental bar.*
- 6.4. Taxol-resistant cells showed Multi Drug Resistance phenotype and resistance to Doxorubicin [1] and Vincristine [2].
 - 6.4.1. LAB MEDIA: Figure 3A. *Video editor: Highlight the pink Taxol-Res response curve*
 - 6.4.2. LAB MEDIA: Figure 3B. *Video editor: Highlight the pink Taxol-Res response curve*
- 6.5. Puromycin selection may not be efficient in resistant cells because puromycin can be pumped out through ABCB1-mediated drug efflux [1].
 - 6.5.1. LAB MEDIA: Figure 3C.
- 6.6. Verapamil alone did not affect cell viability. However, when combined with chemotherapeutic agents, verapamil restored sensitivity to these agents [1]. Non-toxic doses of verapamil also restored puromycin sensitivity in Taxol-resistant cells. [2].
 - 6.6.1. LAB MEDIA: Figure 3E and F *Video editor: Highlight the blue Verapamil curve and compare it with the black Control curve*
 - 6.6.2. LAB MEDIA: Figure 3G and H. *Highlight the blue Verapamil curve on Figure 3H and compare it with the black Control curve.*
- 6.7. When final timepoint samples from DMSO-or Taxol-treated conditions were compared

with the initial timepoint, almost all essential genes were depleted upon knockout, confirming screen quality [1]. In contrast, comparing the DMSO-treated and Taxol-treated endpoint samples revealed no significant gene depletion, as both arms experienced similar selection pressure [2].

- 6.7.1. LAB MEDIA: Figure 4C and D. *Video editor: Highlight the blue points positioned below the center line.*
- 6.7.2. LAB MEDIA: Figure 4E. *Video editor: Highlight the blue points that are clustered close to the center of the plot.*
- 6.8. Multiple positive control genes and epigenetic modifiers were significantly depleted in the EPIKOL (*Epikol*) screen performed in Taxol-resistant cells [1].
 - 6.8.1. LAB MEDIA: Figure 4F. *Video editor: Highlight the colored points located below the dashed horizontal line.*
- 6.9. ABCB1-targeting single guide RNAs were specifically depleted in the Taxol-treated group, confirming ABCB1 as a positive control for Taxol sensitivity in the screen [1].
 - 6.9.1. LAB MEDIA: Figure 4G. *Video editor: Highlight the “Final Timepoint - Taxol” group and the lower counts of ABCB1 single guide RNAs.*

1. Epigenetic
Pronunciation link: <https://www.merriam-webster.com/dictionary/epigenetic>
IPA: /,ep.ə.dʒə'net.ɪk/
Phonetic Spelling: ep·uh·juh·net·ik
2. Chemotherapy resistance
Pronunciation link: <https://www.merriam-webster.com/dictionary/chemotherapy>
IPA: /,ki:.mou'θer.ə.pi ri'zis.təns/
Phonetic Spelling: kee·moh·thair·uh·pee ree·zis·tuhns
3. Triple-negative
Pronunciation link: <https://www.merriam-webster.com/medical/triple-negative>
IPA: /,trip.əl 'neg.ə.tɪv/
Phonetic Spelling: trip·uhl neg·uh·tiv
4. CRISPR
Pronunciation link: <https://www.merriam-webster.com/dictionary/CRISPR>
IPA: /'kris.pə/
Phonetic Spelling: kris·per
5. Multidrug resistance
Pronunciation link: <https://www.merriam-webster.com/dictionary/multidrug>
IPA: /,mʌl.ti'drʌg ri'zis.təns/
Phonetic Spelling: mul·tee·drug ree·zis·tuhns
6. Transduction
Pronunciation link: <https://www.merriam-webster.com/dictionary/transduction>

- IPA: /trænz' dʌk.ʃən/
Phonetic Spelling: tranz·duk·shuhn
7. SUM159PT
Pronunciation link: No confirmed link found
IPA: No verified IPA available
Phonetic Spelling: sum·one·five·nine·pee·tee
 8. HEPES
Pronunciation link: <https://www.howtopronounce.com/hepes>
IPA: /'hep.ez/
Phonetic Spelling: hep·ez
 9. Hydrocortisone
Pronunciation link: <https://www.merriam-webster.com/dictionary/hydrocortisone>
IPA: /, haɪ.droʊ'kɔːr.t̬əˌsoʊn/
Phonetic Spelling: hy·droh·kor·tuh·sohn
 10. Dimethyl sulfoxide
Pronunciation link: <https://www.merriam-webster.com/dictionary/dimethyl%20sulfoxide>
IPA: /daɪ'meθ.əl sʌl'fʌ:k.said/
Phonetic Spelling: dye·meth·uhl sul·fok·side
 11. Luminescence
Pronunciation link: <https://www.merriam-webster.com/dictionary/luminescence>
IPA: /, luː.mə'nes.əns/
Phonetic Spelling: loo·muh·ness·uhns
 12. Nonlinear regression
Pronunciation link: <https://www.merriam-webster.com/dictionary/regression>
IPA: /, nɑːn'lin.i.ə rɪ'ɡref.ən/
Phonetic Spelling: non·lin·eer ree·gresh·uhn
 13. Verapamil
Pronunciation link: <https://www.merriam-webster.com/medical/verapamil>
IPA: /və'ræp.ə.mɪl/
Phonetic Spelling: vuh·rap·uh·mil
 14. Puromycin
Pronunciation link: <https://www.merriam-webster.com/dictionary/puromycin>
IPA: /, pjʊr.oʊ'maɪ.sɪn/
Phonetic Spelling: pyur·oh·my·sin
 15. Lentivirus
Pronunciation link: <https://www.merriam-webster.com/dictionary/lentivirus>
IPA: /'len.t̬əˌvaɪ.rəs/
Phonetic Spelling: len·tuh·vy·ruhs
 16. Blastocidin
Pronunciation link: <https://www.howtopronounce.com/blastocidin>
IPA: /blæs'tɪs.ɪ.dɪn/
Phonetic Spelling: blas·tis·ih·din
 17. sgRNA
Pronunciation link: No confirmed link found

IPA: No verified IPA available

Phonetic Spelling: ess·jee·ar·en·ay

18. Multiplicity of infection

Pronunciation link: <https://www.merriam-webster.com/dictionary/multiplicity>

IPA: /,mʌl.tə'plɪs.ə.ti əv ɪn'fek.ʃən/

Phonetic Spelling: mul·tuh·pliss·uh·tee əv ɪn·fek·shuhn

19. Protamine sulfate

Pronunciation link: <https://www.merriam-webster.com/dictionary/protamine>

IPA: /'prɒʊ.tə'mi:n 'sʌl.fet/

Phonetic Spelling: proh·tuh·meen sul·fayt

20. EPIKOL

Pronunciation link: No confirmed link found

IPA: No verified IPA available

Phonetic Spelling: ep·ih·kol