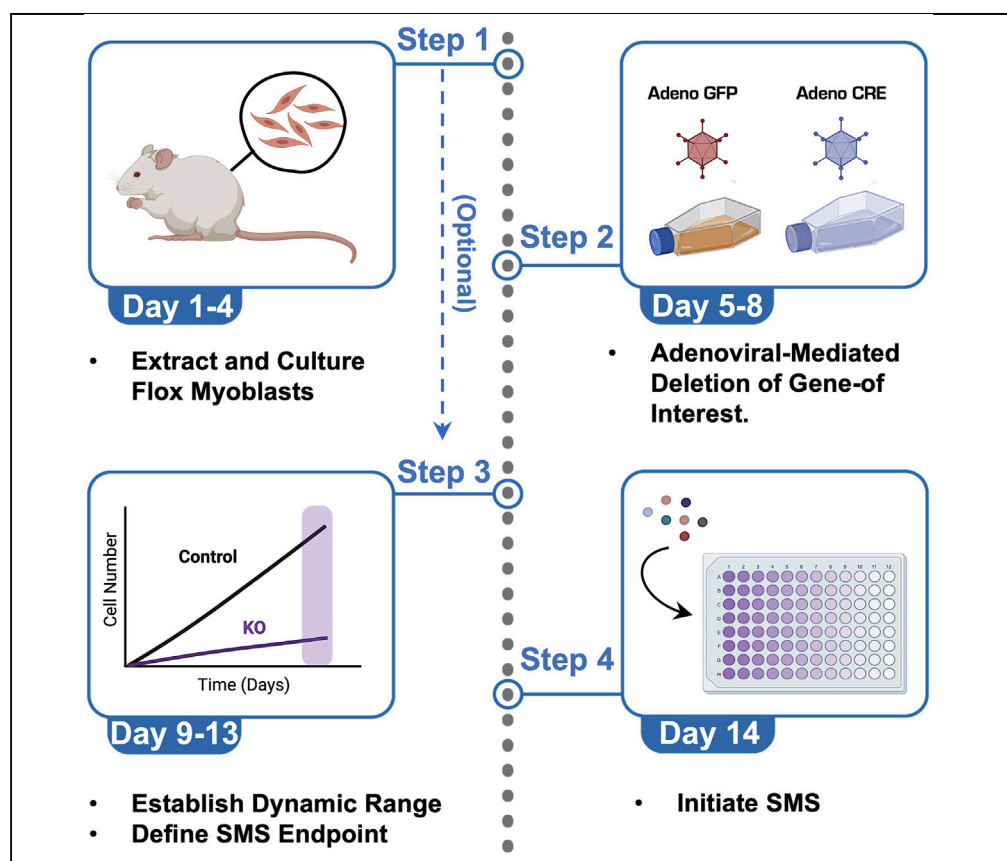


Protocol

Isolation, culture, and use of primary murine myoblasts in small-molecule screens



Small molecule screens (SMS) are often performed using transformed cell lines that have limited physiological relevance to the biological system being investigated, resulting in poor translational outcomes. To circumvent this limitation, we present a protocol to perform SMS in primary murine myoblasts. We describe steps for isolating primary skeletal muscle myoblasts with greater than 95% purity, then describe techniques to establish a robust dynamic range, and conclude with steps to initiate a successful SMS.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Protocol details the isolation of primary myoblasts for use in small-molecule screens

Ensures a robust purity of $\geq 95\%$ myoblasts using the negative selection technique

Guidelines to establish dynamic ranges for *ex vivo* and *in vivo* systems

Highlights multiple scoring criteria options to identify small-molecule positive hits

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Protocol

Isolation, culture, and use of primary murine myoblasts in small-molecule screens

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SUMMARY

Small-molecule screens (SMS) are often performed using transformed cell lines that have limited physiological relevance to the biological system being investigated, resulting in poor translational outcomes. To circumvent this limitation, we present a protocol to perform SMS in primary murine myoblasts. We describe steps for isolating primary skeletal muscle myoblasts with greater than 95% purity, then describe techniques to establish a robust dynamic range, and conclude with steps to initiate a successful SMS.

For complete details on the use and execution of this protocol, please refer to Richler and Yaffe (1970),¹ Rando and Blau (1994),² and Earle et al. (2020).³

BEFORE YOU BEGIN

This protocol will detail the isolation of primary myoblasts from inducible, muscle specific Cox10 knockout mice; however, the method will also be effective for any transgenic or wild type murine models. This protocol enables a comprehensive evaluation of the proliferative and regenerative capacity of myoblasts in response to the administration of small biomolecules. Administration of other factors such as siRNA or CRISPR CAS9 libraries will be equally robust using this protocol and can be administered in a high-throughput manner if access to automated robotic platforms is available to the investigator. The outcome of this primary screening system will identify factors or targets that harbor therapeutic potential to combat muscle mitochondrial disease and/or other muscle-related disorders with high physiological relevance and translational potential.

Institutional permissions

All animal experiments will need to be approved by the Institutional Animal Care and Use Committee of the respective target institution. All animal experiments in this protocol were approved by the Institutional Animal Care and Use Committee at Cornell University in protocol #2020-0104.

Preparation of buffers and culture reagents

⌚ Timing: 20 min

To prepare for the muscle dissection and myoblast isolation you will need Hanks Balanced Salt Solution (HBSS), Dispase and Collagenase II enzymes, Dulbecco's Modified Eagle's Medium/High Glucose (DMEM) (supplied with 10% FBS and 1% Penicillin/Streptomycin (P/S), Ham's F-10 Nutrient Mixture (F-10) (supplied with 20% Horse Serum, 1% P/S) and Recombinant Human basic Fibroblast Growth Factor (bFGF).



1. Coat one T25 flask for each mouse to be dissected with 3 mL of 2% gelatin for at least an hour in a humidified 37°C incubator supplied with 5% CO₂.
2. Add 10 mL of HBSS to a 15 mL conical tube and place on ice. Prepare one tube for each mouse to be dissected.
3. Place DMEM and F-10 media bottles in a 37°C water bath to warm up for 30 min.
4. Ensure that you have a bottle of 70% ethanol and a mouse dissection kit that includes Vannas Scissors, Iris Forceps, Curved Serrated forceps, and Dissecting Scissors.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Pax-7 antibody (1:50)	Santa Cruz Biotechnology	sc-81648
Donkey anti-Mouse IgG secondary antibody, Alexa Fluor 488 (2 drops per mL buffer)	Thermo Fisher Scientific	R37114
Bacterial and viral strains		
Ad5CMVCre	UI Viral Vector Core	VVC-U of Iowa-5
Ad5CMVeGFP	UI Viral Vector Core	VVC-U of Iowa-4
Chemicals, peptides, and recombinant proteins		
Gelatin	Sigma	G-2625
Hanks Balanced Salt Solution (HBSS)	Invitrogen	14170-112
Dispase	Roche	295-825
Collagenase II	Thermo Scientific	17101-015
Dulbecco's Modified Eagle's Medium (DMEM)/High Glucose (4.5 g/L)	Cytiva	SH30243.01
Iscove's Modified Dulbecco's Medium (IMDM)	Gibco	12440-053
Fetal bovine serum (FBS)	Corning	35-010-CV
Penicillin/Streptomycin (P/S)	HyClone	SV30010
Ham's F10 nutrient mixture (F-10)	Gibco	11550-043
Horse serum	Thermo Fisher Scientific	26-050-088
Recombinant human basic fibroblast growth factor (bFGF)	R and D Systems	233-FB-025
bFGF Reconstitution Buffer 2	R and D Systems	RB02
Cell culture grade water	Cytiva	SH30529.02
Hoechst 33342 trihydrochloride trihydrate	Thermo Fisher Scientific	H21492
Propidium iodide	Invitrogen	P1304MP
Trypan blue solution, 0.4% (w/v)	VWR	45000-717
Tamoxifen	Sigma-Aldrich	T5648-1G
Sunflower oil	Sigma-Aldrich	88921-250ML-F
Critical commercial assays		
CellTiter-Glo® 2.0 cell viability assay	Promega	G9242
Experimental models: Organisms/strains		
C57BL/6J, B6.129X1-Cox 10 ^{tm1Ctm} /J	Jackson Labs	Strain# 024697 Female
C57BL/6J, B6.Cg-Pax 7 ^{tm1(cre/ERT2)Gaka} /J	Jackson Labs	Strain# 017763 Male
Oligonucleotides		
FAP forward primer	IDT	GTCACCTGATCGGCAATTTGT
FAP reverse primer	IDT	CCCCATTCTGAAGGTCGTAGAT
Tcf4 forward primer	IDT	CGAAAAGTTCCTCCGGGTTTG
Tcf4 reverse primer	IDT	CGTAGCCGGGCTGATTCAT
Desmin forward primer	IDT	GTTTCAGACTTGACTCAGGCAG
Desmin reverse primer	IDT	TCTCGCAGGTGTAGGACTGG
Pax7 forward primer	IDT	CTCAGTGAGTTCGATTAGCCG
Pax7 reverse primer	IDT	AGACGGTCCCTTTGTCGC
Software and algorithms		
Nexcelom bioscience Celigo 4 channel	Nexcelom	N/A

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
SoftMax Pro 6.2.1	SpectraMax M3	N/A
Other		
Celigo image cytometer	Nexcelom Bioscience	200-BFFL-S
Multi-mode microplate reader	SpectraMax M3	N/A
VWR shaking water bath	VWR	10128-126
Falcon® tissue culture flasks T25 flasks	VWR	29185-300
Falcon® tissue culture flasks T75 flasks	VWR	BD353136
Zeiss Axio Observer Z1 fluorescence microscope	Zeiss	Axio Observer Z1
Mouse dissecting kit	WPI	MOUSEKIT
96 well tissue culture plates	VWR	82050-771
E1-ClipTip multichannel pipettor	VWR	75876-180
Insulin syringe	VWR	BD324702
Ultrasonic cleaner	VWR	Model# 97043-988
Diagenode SA Bioruptor Plus	Diagenode	B01020001
Cell strainer 100 µm sterile	VWR	76327-102
Cell strainer 40 µm sterile	VWR	76327-098

MATERIALS AND EQUIPMENT

Prepare all buffers and media according to the tables. Refer to the [key resources table](#) and [materials and equipment](#) for a complete list of reagents, and equipment.

△ **CRITICAL:** HBSS should be kept on ice when performing the muscle isolation and collection. Store Dispase and Collagenase II solutions in the freezer in 2 mL aliquots to prevent freeze thaw cycles. bFGF should be stored in 10 µL aliquots at -20 degrees until use. Warm Dispase and Collagenase II solutions and media in a 37°C water bath before use.

Dispase II stock

Reagent	Stock concentration	Final concentration	Amount
HBSS	1×	1×	99.75 mL
Dispase	Varies	2.4 U/mL	240 U
Calcium Chloride	1 M	2.5 mM	250 µL
Total			100 mL

Store 2 mL aliquots in the -20°C until use. Stocks are stable for 12 months.

Collagenase II stock

Reagent	Stock concentration	Final concentration	Amount
HBSS	1×	1×	1997.5 µL
Collagenase II	Varies	3,600 U/mL	7,200 U
Calcium Chloride	1 M	2.5 mM	2.5 µL
Total			2 mL

Prepare fresh at the time of hindlimb dissection and store on ice until use.

D10

Reagent	Stock concentration	Final concentration	Amount
DMEM High Glucose (4.5 g/L)	1×	1×	445 mL
FBS	N/A	10%	50 mL
P/S	100×	1%	5 mL
Total			500 mL

Store in 4°C. Stock media is stable for 12 months.

Primary myoblast growth medium (PMGM)			
Reagent	Stock concentration	Final concentration	Amount
F10	1×	1×	395 mL
Horse Serum	N/A	20%	100 mL
P/S	100×	1%	5 mL
bFGF	250 µg/mL	25 ng/mL	Supply at the time of use based on the volume of media for plating
Total			500 mL

Store in 4°C, stock media is stable for 12 months. At the time of use, warm to 37°C, take 5 mL (for each T25 flask) or 15 mL (for each T75 flask) and supplement with bFGF (diluted 1:10000 from bFGF stock) to keep myoblasts in the proliferative state. Media with bFGF can be stored in 4°C and used up to one week.

- **250 µg/mL bFGF solution:** add 25 µg bFGF Powder in 100 µL Reconstitution Buffer 2 (0.1% Bovine Serum Albumin in PBS).

Store 10 µL aliquots in –20°C, Stocks are stable for 1 month.

- **2% Gelatin:** add 2 g gelatin Powder in 100 mL ddH₂O.

Autoclave the 2% gelatin solution, then store in 4°C. Solution will solidify at 4°C. Before coating, warm up the solution in a 37°C water bath until gelatin is completely liquidized. Gelatin is good for 3 warm-up cycles.

- **75 mg/mL Tamoxifen Stock Solution for IP Injections:** add 75 mg tamoxifen Powder in 1 mL 100% Ethanol.

Dilute tamoxifen in 100% ethanol to make a final stock concentration of 75 mg/mL. Vortex and then sonicate for 30 min at 37°C (if using the Ultrasonic Cleaner) or 20 cycles with 60 s on and 10 s off (if using the Bioruptor).

- **10 mg/mL Tamoxifen Stock Solution for IP Injections:** Transfer 133 µL of 75 mg/mL tamoxifen stock solution to 867 µL of filtered sunflower oil.

Store in 4°C (solution is stable for 7 days). Each day before injection, vortex the tamoxifen/sunflower oil mixture until clear and heat at 37°C for 45 min. Inject 3 µL/g of mice bodyweight.

- **10 mg/mL Hoechst 33342:** add 100 mg hoescht Powder in 10 mL ddH₂O.

Store 1 mL of Aliquots at 4°C. Stocks are stable for 2 years.

- **1 mg/mL Propidium iodide Solution:** add 10 mg Propidium iodide Powder in 10 mL ddH₂O.

Store 1 mL of Aliquots at 4°C. Stocks are stable for 2 years.

STEP-BY-STEP METHOD DETAILS

Tissue isolation and digestion

⌚ Timing: ~2 h

These steps include dissection of the muscle tissue from the hindlimb of 3–5-week-old male mice, followed by mechanical and enzymatic digestion to liberate isolated myoblasts. Using mice 3–5 weeks old will ensure a robust yield of myoblasts. Older mice can be used but the myoblast yield

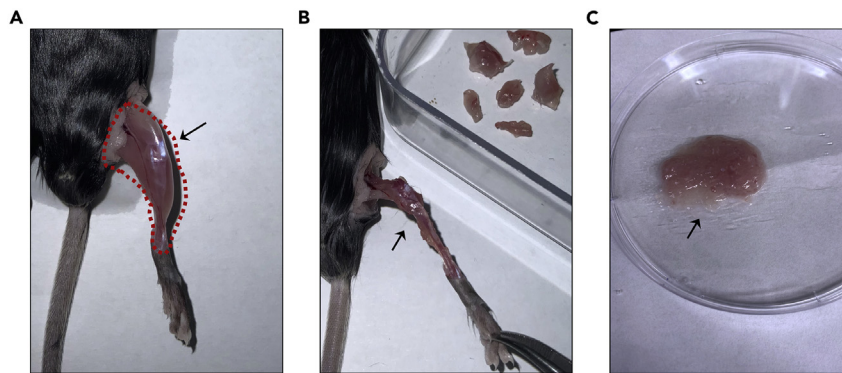


Figure 1. Dissection of muscle tissue from the mouse hindlimb

(A) Exposed hindlimb muscle tissue.

(B) Representative example of a thorough dissection of muscle tissue from the hindlimb.

(C) Tissue pulp after mechanical shearing with dissecting Vannas scissors performed before exposure to digestion enzymes and shaking.

will significantly decrease with age. A series of purification steps will then take place to yield a purified myoblast culture.

1. Dissect mice at 4 weeks of age.
 - a. Euthanize the mouse by CO₂ asphyxiation and spray the hind limb area with 70% ethanol.
 - b. Make a horizontal incision on the lower abdominal area using Vannas scissors.
 - c. Pull down the skin to expose the hindlimb muscle ([Figure 1A](#)).
2. Use dissecting scissors to cut off as much muscle tissue as possible (highlighted in [Figure 1B](#)).
 - a. Using curved, serrated forceps, place the harvested tissue in a 15 mL conical tube of HBSS on ice until ready to perform digestion steps (Repeat this step for the second hindlimb).
3. To begin digestion, discard the HBSS from the isolated muscle tissue, wash once with fresh HBSS and place on a petri dish on ice.
 - a. Mechanically shear the tissue with dissecting Vannas scissors until you obtain a paste-like texture ([Figure 1C](#)).
4. Transfer mixture to a clean, pre-weighed 15 mL conical tube and weigh the tissue.
5. Add 2 mL of Dispase II and 2 mL of Collagenase II enzyme solutions for each gram weight of tissue.
6. Place the tube into a shaking water bath set to a speed of 150 strokes/min at 37°C for 20 min to begin the enzymatic digestion.
 - a. After 20 min pipette the solution up and down at medium speed using a 10 mL pipette 20 times to assist with the dissociation of the tissue while leaving the cells intact.
 - b. Return to the water bath for another 10 min.
7. Remove the tube and pipette up and down 20 times using a 5 mL pipette.
8. Add 4 mL of DMEM supplemented with 10% FBS and 1% Penicillin/Streptomycin (D10) to the enzyme cell mixture and filter the entire content through a 100 μ M sterile filter into a 50 mL conical tube to remove large debris and undigested tissue.
9. Next, filter the flow-through, through a 40 μ M sterile filter into a clean 50 mL conical tube.
10. Centrifuge at 800 $\times$ g for 5 min to pellet the cells.
11. Aspirate the supernatant and wash the remaining cell pellet in 4 mL D10. Centrifuge once again at 800 $\times$ g for 5 min to pellet.
12. Aspirate the supernatant and wash the remaining cell pellet in 4 mL of D10.
 - a. Transfer the cell mixture to a clean 15 mL conical tube.
 - b. Repeat washes with 4 mL of D10 three times for a total of five washes. On the final wash, centrifuge at 300 $\times$ g for 5 min.
13. Resuspend pellet in 5 mL of primary myoblast growth medium (PMGM growth medium) supplemented with fresh bFGF at a concentration of 1:10,000.



Figure 2. Schematic highlighting dislodged primary myoblast capture

- a. Aspirate gelatin from the pre-coated T25 flask (from step 1), and plate cell resuspension.
- b. Culture for 48 h for cellular expansion in a humidified 37°C incubator supplied with 5% CO₂. At this step, myoblasts are considered to be at passage zero (P0).

Purification and maintenance of primary myoblasts

14. After 48 h of expansion, coat a new T25 flask with 2% gelatin for at least an hour and incubate PBS and primary myoblast growth medium in a 37°C water bath for 30 min.
15. Remove the culture flask from the incubator and gently aspirate the culture medium and add 3 mL PBS to the flask and let it sit at room temperature for 2 min.
16. After 2 min perform the negative selection purification method which takes advantage of the weak adherence properties of the myoblasts compared to fibroblast.
 - a. Hold the cell culture flasks firmly in your hand and subject it to a strong mechanical force by performing five firm taps gently hitting the flask against a hard surface on each side to dislodge the myoblasts while leaving the fibroblasts attached to the plate surface.
 - b. When complete, visualize cells under the microscope to confirm myoblast detachment (suspended small rounded circular cells). Approximately 70% of the myoblasts will be detached.

△ CRITICAL: The remaining 30% of myoblasts will remain attached to the flask. This is expected and they should be left behind to ensure a pure myoblast culture. If you attempt to increase the mechanical force to dislodge all myoblasts, the risk of unwanted cell type transfer is high and will decrease the purity of the culture.

17. Carefully add 3 mL primary myoblast growth medium to the culture flasks with the PBS and dislodged myoblasts being mindful not to scrape the cell culture surface and transfer the entire content to a 15 mL conical tube (Figure 2). Centrifuge the suspended myoblasts at 300 ×g for 5 min to pellet the cells and aspirate the supernatant.

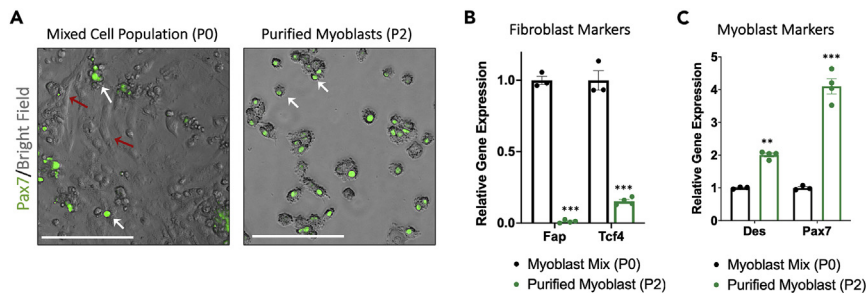


Figure 3. Characterization of purified myoblasts

(A) Representative images showing the crude culture with a heterogeneous cell population (e.g., RBCs, fibroblast, myoblast) 48 h after plating (left) and purified culture of ~90% myoblasts after two passages (right). Cells were fixed and immunostained for Pax7. Images were merged with Pax7 and phase contrast. Red arrows indicate fibroblasts without Pax7 stain. White arrows indicate myoblasts with Pax7 stain. Scale bars = 200 μ m.

(B and C) Expression of fibroblast markers (*Fap* and *Tcf4*) and muscle markers (*Desmin* and *Pax7*) genes normalized to the house keeping gene *RPL13* in heterogeneous cell population and purified myoblast. Data represent mean $\pm$ SEM. * is p-value < 0.05, ** is p-value < 0.01 and *** is p-value < 0.001 by Student's T test.

△ CRITICAL: When collecting dislodged myoblasts, avoid touching or scraping the cell flask surface (where the cells are adhered) as this may dislodge any unwanted cell types and introduce it to the myoblast culture (Figure 2).

18. Resuspend the cell pellet in 5 mL of PMGM media supplemented with bFGF and return to the incubator for continued expansion.
19. Myoblast growth cultures are then maintained and split every 48 h using a 1:10 Trypsin to PBS solution to continue to take advantage of the differential adherence properties of the purified myoblast vs fibroblast.

Note: A greater than 95% myoblast purity will be achieved after passage 2 with a 1:10 trypsin split (Figure 3). After P2, myoblasts will be ready to move to a T75 for continual expansion.

△ CRITICAL: To ensure that the myoblasts remain in the proliferative state, always supplement the culture media with fresh bFGF when plating after each low trypsin split and ensure that the confluency does not exceed 50%. The bFGF will be stable in culture for up to 5 days.

Note: All myoblasts cultured and expanded prior to the 1:10 low trypsin:PBS split are considered to be at passage 0. Every subsequent low trypsin:PBS split will increase the passage. Myoblasts perform well in culture up until passage 6 (P6). Primary myoblasts should be cryopreserved at a very early passage (P1-P2) for future SMS applications. Approximately 90% of the primary myoblasts will be recovered after cryopreserved cells are thawed.

Note: Purified myoblast cultures can be visualized morphologically by eye or confirmed by extracting RNA and documenting a decline in fibroblast markers and an enrichment of myoblast marker genes such as *Desmin* or *Pax7* (Figures 3B and 3C).

Small molecule screening to assess regenerative capacity of myoblasts

Option 1: Ex vivo knockout primary myoblasts: Adenoviral CRE-mediated excision of gene of interest (GOI)

⌚ Timing: 4 days

This step will detail the preparation of an adenoviral CRE-mediated gene knockout in myoblasts for SMS to assess myoblast regenerative capacity as the primary readout. Myoblasts isolated from

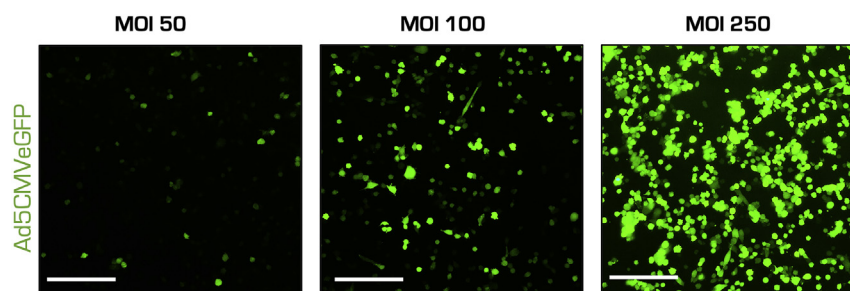


Figure 4. MOI of primary myoblasts with Ad5CMVeGFP

Myoblasts were transduced with Ad5CMVeGFP at a MOI of 50 (Left), MOI of 100 (Middle) and MOI of 250 (Right) following steps 19–22. After 3 days, live myoblasts were imaged under fluorescent microscope. Scale bars = 200 μ m.

C57BL/6J mice with floxed alleles of Cox10 will be used as an example in this protocol but any floxed transgenic mouse model will be effective.

20. Now that a purified myoblast culture has been obtained following steps 1–19, perform a 1:10 low trypsin: PBS split of the floxed myoblasts.
 - a. Seed myoblasts in PMGM growth media at a concentration of 4×10^5 cells on a T75 gelatin-coated flask in 15 mL PMGM media.

Note: You will need two T75 flasks: one for Adeno GFP infection and the other for the Adeno CRE infection.

21. Culture for 24 h in an incubator at 37°C with 5% CO₂.
22. After 24 h, begin the preparation of the adenovirus infection.
 - a. First, calculate the desired Multiplicity of Infection (MOI) for your gene target (please see the critical section for how to perform this calculation).
 - b. For the ex vivo deletion of Cox10 in primary myoblasts, an MOI of 250 was used.
 - c. Dilute 2.5 μ L of stock GFP-expressing or Cre-expressing adenovirus (a titer of 4×10^{10} pfu/mL) to 1 mL pre-warmed PMGM growth medium.
 - d. Add directly to the 15 mL myoblast T75 culture flask and gently shake the flask to evenly distribute the adenovirus into the culture media.
23. Return the infected cell flask to the incubator and culture for an additional 72 h.
24. To check for infection efficiency, visualize the Adeno GFP expression using a fluorescent microscope after 72 h of viral incubation time.

△ CRITICAL: Myoblast should have 100% penetrance of the Adeno GFP viral expression (Figure 4). If this is not the case, then increase the MOI.

25. Aspirate the viral cell culture media and rinse cells gently with 3 mL of PBS.
 - a. Remove the PBS, then trypsinize the virus treated myoblasts using 2 mL of a 1:10 Trypsin to PBS solution.
26. Collect the trypsinized cells and place in a 15 mL conical tube and centrifuge at 300 $\times$ g for 5 min to pellet. Following the spin, aspirate the supernatant.
27. Take an aliquot of 1×10^5 myoblasts from the Adeno GFP and Cre treatment groups and return the remaining cells to the incubator to continue with cellular expansion.
 - a. Centrifuge Adeno GFP and CRE myoblast aliquots at 300 $\times$ g for 5 min to obtain cell pellets.
 - b. Confirm the protein target knockout in these cells using western blot and/or qPCR with appropriate target antibodies and/or primers.
28. After confirming your GOI is successfully ablated, primary myoblasts can be used to establish a SMS dynamic range. See “Establishing the dynamic range” section.

△ **CRITICAL:** MOI is defined as the ratio of infectious agents to cells. Therefore, if 1×10^6 viral particles were added to 1×10^6 primary myoblasts with the assumption that each viral particle infected a single cell, then the MOI would be 1. The MOI needs to be empirically determined according to your GOI by both evaluating infection efficiency by using Adeno GFP penetrance as a proxy (Figure 4) in addition to performing standard immunoblotting techniques to determine if your GOI is ablated when infected with Adeno CRE. To establish an *ex vivo* KO of Cox10 in primary myoblasts, we utilized an MOI of 250.

To calculate the amount of virus needed after establishing MOI:

(Total number of myoblasts per flask or well) $\times$ (desired MOI) = total viral particles or plaque forming units (pfu) needed.

(Total pfu needed) / (pfu/mL functional titer) $\times$ 1000 = total μ L of adenoviral particles to add to each flask or well.

Example calculation: For 1×10^5 of myoblasts, MOI of 250, and 4×10^{10} pfu/mL functional titer of stock adenoviral.

Total pfu needed = $1 \times 10^5 \times 250 = 250 \times 10^5$ pfu.

Total volume of adenoviral particles = 250×10^5 pfu / 4×10^{10} pfu/mL $\times$ 1000 = 0.625 μ L of adenoviral particles.

When handling adenovirus, please use the appropriate safety guidelines to both handle and discard all contaminated materials.

Option 2: *In vivo* knockout primary myoblasts: Tamoxifen-induced CRE-mediated excision of GOI

⌚ **Timing:** 2 weeks

In lieu of knocking out your GOI *ex vivo* using adenovirus administration, this step will detail the isolation and purification of myoblasts from knockout mice that have already been exposed to the CRE recombinase *in vivo*. In this example, myoblasts will be isolated from the satellite cell-specific Cox10^{Flox/Pax7CRE-ERT2} mice to assess myoblast regenerative capacity as the primary readout in SMS. Mice are intraperitoneally injected with tamoxifen to induce the *in vivo* Cre-mediated excision of Cox10.

29. Weigh two 4-week-old male Cox10^{Flox/Pax7CRE-ERT2} mice and administer either tamoxifen (KO group) or vehicle (control group) via intraperitoneal injections (IP) at a concentration of 30 mg/g body weight daily for 5 days.

Note: After the 5th day of IP injection, leave mice undisturbed for 7 days with no injections. ("washout" period).

30. Euthanize the mice with CO₂ asphyxiation and isolate primary myoblasts and maintain in culture as detailed above in the "tissue isolation and digestion" section (steps 1–19).

31. Confirm the knockout using Western blot and qPCR as described in step 27.

Establishing the dynamic range for small molecule screens

This step will define the dynamic range between the baseline proliferation capacity of healthy control myoblasts compared to your GOI knockout myoblasts. This step is critical to establish the rescue

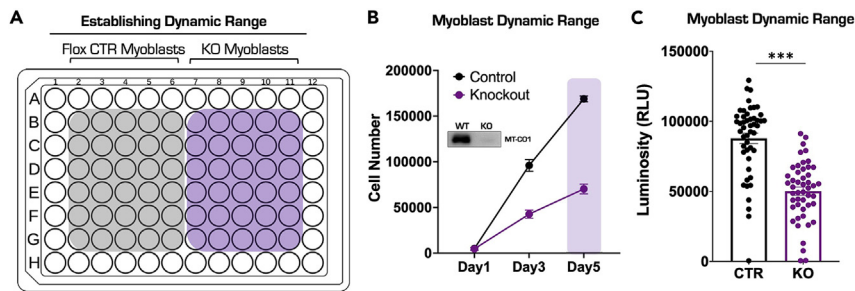


Figure 5. Establishing primary myoblast dynamic range for HTS

(A) Schematic representation of the plate layout to evaluate growth dynamics between knockout and control myoblasts.

(B) Representative example of dynamic growth difference between Cox10 knockout and Flox control myoblasts in a 24-well format over five days using Celigo. Inlay is a WB demonstrating successful KO of MT-CO1 (a proxy for Cox10). Data represent mean $\pm$ SEM.

(C) End point dynamic growth dynamic difference between Cox10 knockout and flox control myoblasts using Cell Titer Glo assay. A ≥ 2 -fold dynamic range is considered a successful for rescue in a SMS screen. Data represent mean $\pm$ SEM. *** is p-value < 0.001 by Student's T test.

potential of your SMS. This step is performed after validating the knockout efficiency and prior to initiating a screen.

32. Coat three 96-well plates with 100 μ L of 2% gelatin per well for at least 1 h.

Note: Each plate will be dedicated to a Day 1, Day 3, or Day 5 timepoint evaluation when establishing the dynamic range.

33. After an hour, remove the 2% gelatin from 96-well plates.

34. Trypsinize a T-75 flask of primary myoblasts using the low trypsin split as described previously.

a. Seed 30 wells of control and 30 wells of knockout myoblasts in 200 μ L/well of PMGM growth media at a concentration of 2.4×10^3 cells/well according to the layout in [Figure 5A](#).

b. After seeding, leave plates in the cell culture hood for 15 min at 37°C to allow myoblasts to briefly settle before placing them in the incubator.

35. Culture for 24 h in an incubator at 37°C with 5% CO₂.

36. Following the 24 h incubation time, prepare the staining buffer.

a. First, calculate the amount of staining buffer needed: number of primary myoblast wells $\times$ 100 μ L per well.

b. Next, prepare the staining buffer by adding 0.5 μ L of Hoechst stock solution and 2 μ L of PI stock solution per 1 mL of pre-warmed serum free D10 media.

37. Add staining buffer to the myoblasts.

a. Slowly remove 180 μ L of culture medium from the cell plates with a multichannel pipette leaving behind 20 μ L so that the primary myoblasts do not dry.

b. Add 100 μ L of staining buffer per well to the myoblasts.

38. Return the plate to the incubator and incubate for 45 min at 37°C with 5% CO₂.

39. Remove the cell plate from the incubator and perform the cell count using an automated cell counting instrument. As an example, we used the Nexcelom Celigo cytometer.

a. Record the myoblasts count labeled with Hoechst (Blue channel) and PI (Red Channel).

b. Then calculate the live cell number as: Hoechst-stained cell number – PI-stained cell number for the control and KO myoblasts. This will be the dynamic range obtained after one day of culture.

40. Replace medium in the remaining two 96-well plates.

a. Slowly remove 180 μ L of culture medium with a multichannel pipette leaving 20 μ L behind.

- b. Replace it with 180 μ L of fresh PMGM growth media.
- c. Incubate for an additional 48 h.
41. Repeat steps 35–40 and record the dynamic range of the control and knockout myoblasts for the Day 3 and Day 5 timepoints (Figure 5B).
42. Determine which culture timepoint gives the maximal dynamic range.

Note: A ≥ 2 -fold dynamic range would be considered successful for rescue potential in a screen. For COX10 KO primary myoblasts, the maximal culture dynamic range was achieved at 5 days and is the endpoint of the SMS (Figure 5C).

△ CRITICAL: Never aspirate culture media using a vacuum manifold when in a 96-well format. The weak adherent properties of myoblasts will cause the cells to dislodge.

Note: Place 200 μ L of PBS in the wells surrounding the perimeter of the 96-well plate to minimize edge effects.

Note: Control myoblasts should reach 80% confluency on Day 5, if longer culture time is required, seeding density should be adjusted accordingly.

Note: Other cellular quantification methods such as the Cell Titer Glo can be utilized to establish the dynamic range in Figure 5B.

Initiating a small molecule screen in primary myoblasts

This step will detail the initiation of a small molecule screen in primary myoblasts using cellular regenerative capacity as the primary readout. Although the use of small molecules is highlighted in this section, employing siRNA or CRISPR/Cas9 libraries are also robust options. Furthermore, other readouts such as tagged proteins or fluorescent expression constructs will be equally effective utilizing these methods. This step is initiated after the appropriate dynamic range and experimental culture endpoints have been established.

43. Coat two 96-well plates with 2% gelatin according to the guidelines outlined in step 32.

Note: Each plate will be an independent screen replicate to ensure SMS rigor and reproducibility.

44. After 1 h, remove the gelatin.
45. Seed 2.4×10^3 flox control and knockout myoblasts per well in 100 μ L of PMGM supplemented with bFGF according to the plate layout outlined in Figure 6.
46. Incubate myoblasts for 24 h at 37°C with 5% CO₂.
47. Following incubation, expose the KO myoblasts to a unique small biomolecule by administering directly into the well.

Note: Compound concentration must be determined empirically but a typical starting concentration for SMS applications is 10–25 μ M. Small biomolecules are tested in duplicate following the plate layout in Figure 6 to allow for the comprehensive profiling for up to 27 small biomolecules per plate.

48. After compound administration, gently swirl the cell culture plate to ensure even distribution.
49. Return the plates to the incubator to culture for 5 days or according to your established experimental endpoint defined in section “Establishing Dynamic Range”.
50. After 5 days, assess the rescue potential of the small biomolecules by measuring myoblast regenerative capacity as described previously following steps 35–39.
51. Analyze the data.

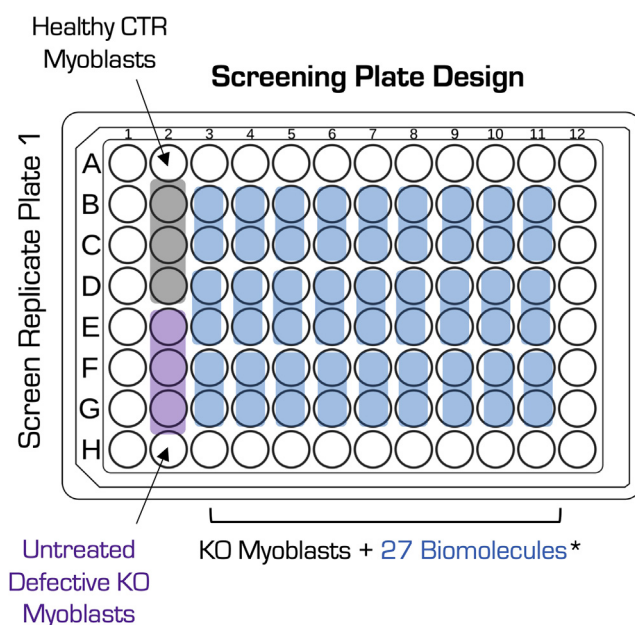


Figure 6. Seeding plate layout for SMS

- Calculate the fold rescue potential of each small biomolecule for both screen replicate plates according to the equation detailed in [Figure 7A](#).
- Then plot data from both SMS replicates 1 and 2 against each other to define Pearson's correlation coefficient ([Figure 7B](#)).

Note: This will assess rigor and reproducibility of the SMS. Compounds that yield a 30% rescue (1.3-fold) or greater for both screen plates are scored as positive biomolecules for downstream analyses, but this can be empirically defined according to your specific application.

△ CRITICAL: Compounds are only scored positive if they cross the fold-change threshold on both screen plate replicates ([Figure 7B](#)). That said, be mindful of false-positive and false-negative results. It is heavily encouraged to closely evaluate the original values for all 4 candidate replicate values to identify potential outliers that may influence and sway the fold change calculations.

Note: Positive lead biomolecules candidates must be validated by performing 1) downstream concentration dose response analyses to identify the optimal drug concentration and 2) time course analyses to define drug dynamics.

Note: As cell proliferation is a proxy for myoblast regenerative capacity, it is critical that myoblasts are evenly seeded at the beginning of the assay. A cell count 3 h post seeding in step 43 using the phase-contrast scan on an automated cell counter is recommended. These values can then be used to confirm that cells were evenly seeded and can be further for normalization purposes.

Note: If the investigator has access to automated robotic seeding and dispensing equipment platforms such as the Thermo-Fisher Combi Reagent Dispenser and the Agilent Bravo Automated Liquid Handler, then step 42 can be advanced to a high-throughput screening (HTS) capacity. The modification to HTS is as follows:

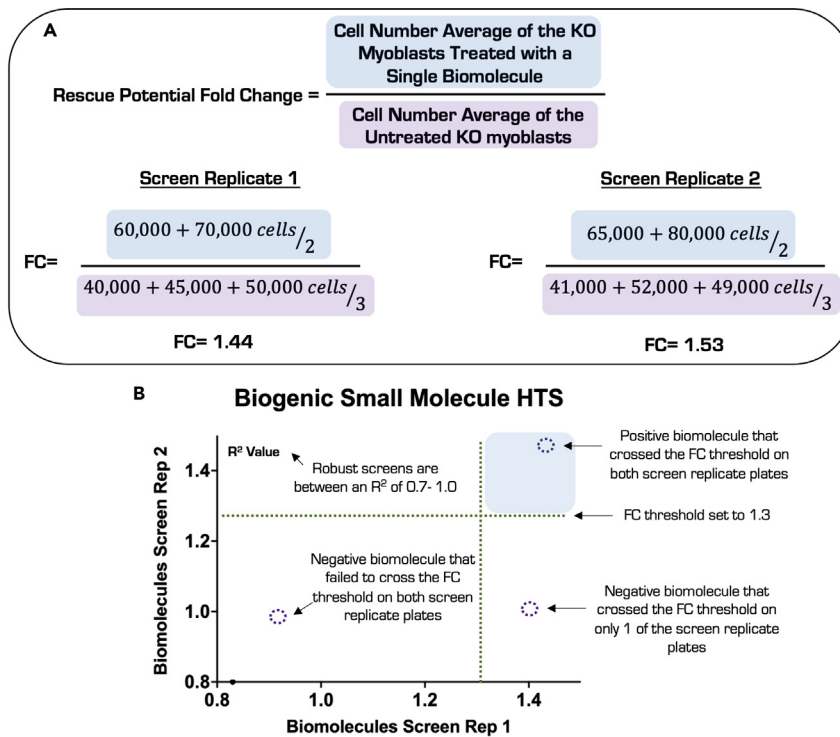


Figure 7. Analysis screen result using fold change

(A) Equation to calculate rescue potential fold change (top), and two representative examples of how to apply the equation with two screen replicates.

(B) Schematic representation of an X-Y plot of two replicate biogenic small molecule screen. X axis, Biomolecules screen replicate 1. Y axis, Biomolecules screen replicate 2. FC threshold was set to 1.3. Negative biomolecules that failed to cross the FC threshold on both screen replicate plates are located at lower left quadrant or that crossed the FC threshold on only one of the screen replicate plates are located at the upper left quadrant and lower right quadrant. Positive biomolecules that crossed the FC threshold on both screen replicate plates are located at the upper right quadrant.

Step 42- Increase the number of 96-well culture plates as desired. For typical HTS, approximately 30–40 plates are seeded at a time.

EXPECTED OUTCOMES

This protocol is suitable for myoblasts harvested from any wild type or transgenic mouse model and can be leveraged towards most screen applications such as the administration of small molecules or CRISPR-Cas9 libraries. The primary myoblasts yield following purification is $\geq 95\%$ (Figure 3). Myoblasts can then be expanded and frozen down at an early passage and can be robustly employed until passage 6. To initiate a successful SMS, a dynamic growth range of ≥ 2 -fold between experimental groups is required (Figure 5). Successful completion of this protocol will yield a positive small molecule hit rate of 1% as is predicted for HTS.^{4,5} Unlike most SMS however, these positive hits will harbor extreme physiological relevance to muscle biology as they were screened directly in the affected primary system. The therapeutic potential of these positive candidates can then be defined against a wide range of myopathies in further downstream analyses.

QUANTIFICATION AND STATISTICAL ANALYSIS

Option 1: Defining rescue potential based on the fold change (FC)

A fold change rescue potential calculates how well each biomolecule can restore regenerative potential above the growth levels of the *KO myoblast controls*.

- Calculate the fold change rescue potential of each screen plate by taking the average cell number of the KO myoblasts treated with a single biomolecule and dividing it by the average cell number of the **untreated knockout myoblast controls** (Figure 7A).
- Next, plot the FC of each compound on screen replicate 1 against the corresponding FC calculated from screen replicate 2 on an X-Y plot (Figure 7B).
- A cutoff point can then be set empirically according to your experimental system. A fold change rescue of 1.3 (30%) or greater is considered robust.

Option 2: Defining rescue potential based on the percentage of healthy myoblast controls

A percentage rescue potential calculates how well each biomolecule can restore regenerative potential back to growth levels of **healthy myoblast controls**.⁶

- Calculate the percentage rescue potential of each screen plate by taking the average cell number of the KO myoblasts treated with a single biomolecule and dividing it by the average cell number of the **healthy myoblast control group**, then multiplying that value by 100.
- Next, plot the percentage rescue potential of biomolecules on screen replicate plate 1 against the corresponding values obtained from screen replicate plate 2 on a X-Y plot.
- Positive biomolecules can then be scored as compounds that were able to successfully rescue myoblast regenerative capacity back to 70% of wildtype myoblast control levels. Note that this percentage cutoff can be empirically defined according to your experimental system.

Option 3: Defining rescue potential based on Z scores

Rescue potential can also be defined by calculating the Z scores of each biomolecule as described in Soustek et al.⁷

- First, calculate the Z score for each individual compound tested using the following formula:

$$Z' = (x - \mu) / \sigma$$
 - x: the cell number from the knockout myoblasts after treatment of a single compound.
 - μ : the cell number mean of all **treated knockout myoblast controls**.
 - σ is the standard deviation of all **treated knockout myoblast controls**.
- The Z score will provide the standard deviation of each small biomolecule and determine how far away it is from the reference population mean (**treated knockout myoblast controls**). As we are examining rescue potential, positive Z values are desired and appropriate cut-offs are empirically defined according to your experimental system. As an example, in Soustek et al.,⁷ a Z score between 1.8 – 3.0 was considered a weak hit and > 4.0 was considered a strong hit.

LIMITATIONS

One limitation of this protocol is that primary cells cultures are restricted to a short time frame in culture (~2weeks) and experience reduced integrity at extended passages. To overcome this limitation, as mentioned above (see critical section after step 19), refrain from using cells beyond passage number 6. Ensure that cells are frozen down at a very early passage (P2) for later use in the SMS (see critical section following step 19 in the “purifying and maintaining primary myoblast” section). Considering these limitations, a robust mouse colony is imperative to ensure a constant supply of myoblasts available for cryopreservation and culture.

TROUBLESHOOTING

Problem 1

Poor myoblast yield during muscle tissue digestion (steps 1–13).

Potential solution

The isolation and digestion of both hindlimbs from a single 4-week-old mouse should result in a robust yield of primary myoblasts and provide enough material to seed four screen plates to assay 54 small molecules in duplicate. If muscle digestion results in a poor myoblast yield, first ensure that

the mice are within 3–5 weeks of age as the myoblast yield is the most robust at this stage. Also ensure that digestion and isolation reagents are prepared fresh and both mechanical and enzymatic digestion steps are followed. If yield is still poor, it is possible to increase the input material by dissecting the hindlimbs of an additional mouse and pooling the cells. If this option is selected, please be mindful of the enzyme to tissue ratio to ensure thorough digestion (step 5).

Problem 2

Retained fibroblasts following PBS split (steps 14–19).

Potential solution

The negative selection technique for purifying the myoblasts as mentioned above (step 16) takes advantage of the low adherence properties of the myoblasts relative to fibroblasts. However, fibroblasts may occasionally lift during the mechanical disassociation procedure (step 16) and be carried over into the new culture. If too many fibroblasts transfer, their increased growth rate will overtake the culture. To rectify this, follow up with a second PBS split in 48 h. This should resolve the issue. Additionally, one round of tapping on each side is enough for myoblast disassociation (step 16). Any more than this will risk carry over of fibroblast into the purified culture.

Problem 3

Failure of knockout myoblasts to proliferate in parallel with controls (steps 32–41).

Potential solution

Genetic knockout will often lead to reduced cell viability. As a result, when culturing knockout cells and controls, knockout myoblasts may grow significantly slower than the controls which means their passage time will be different. To overcome this during the expansion phase, adjust seeding density accordingly. As an example, one could double the concentration of the knockout myoblasts relative to controls to ensure that both experimental groups grow at a similar passage until you are ready to seed for the experiment. Additionally, do not forget to supplement the media with bFGF as it is critical for the activation of the myoblasts and to keep them in a proliferative state.

Problem 4

Failure to establish a large enough dynamic range (steps 32–41).

Potential solution

A successful dynamic range (≥ 2 -fold difference) is necessary before initiating a screen. If knockout cells are not growing slower than controls, consider introducing a stress condition. This will be determined by the type of screen being performed and the intended readout. In the case of this protocol, genetic ablation COX10 (a key player in oxidative phosphorylation) compromises energy production. An effective stress condition therefore would be to reduce the nutrient levels (glucose) in the growth medium since this stress would force dependence on energy production via the oxidative phosphorylation pathway and as a consequence will amplify the dynamic range between experimental groups.

Problem 5

Screening efforts do not yield positive lead hits (step 49).

Potential solution

The predicted hit rate for HTS small molecule screens is 1%. Therefore, if a screen library is composed of 100 small molecules, only one is predicted to score positive on the screen. If no positive hits are identified, it is possible that the compound library is too small and should be increased to at least 500–1,000 compounds to ensure that the 1% hit rate is being captured. Additionally, in this example, a 30% increase in myoblast regenerative capacity was the selection criteria for a

positive hit. This however needs to be determined empirically according to the investigator's biological system. It is possible that a biologically significant rescue can be achieved with a less stringent cutoff.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Joeva Jade Barrow (jb2254@cornell.edu).

Materials availability

This study did not generate any unique materials or reagents.

Data and code availability

This study did not generate any unique datasets or code.

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AUTHOR CONTRIBUTIONS

Conceptualization, investigation, data acquisition, and writing were performed by Y.Q., K.E., and J.J.B. Funding acquisition and supervision were performed by J.J.B.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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