RoosterBio®

Mesenchymal Stem Cell Boost Protocol

For research scale[1] mechanoporation of mesenchymal stem cells

1. Product Description

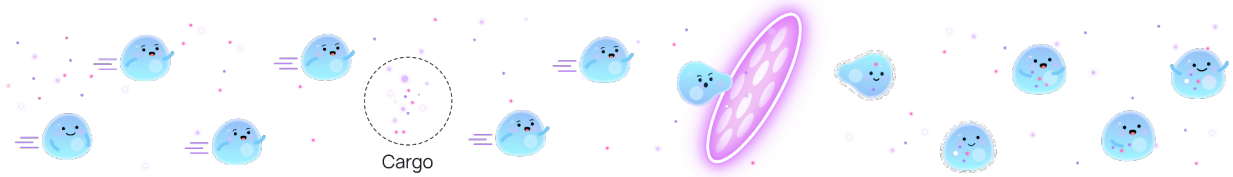
Deliver your desired cargo[2] to a population of mesenchymal stem cells (MSCs) using Portal's technology. Mechanoporation with Portal, or Boosting, is simple, fast, and has minimal impact on cell state, enabling a broad range of research and clinical applications.

[1] 50-200 µl volume

[2] Cargo options may include but are not limited to fluorescent polymers (dextran), mRNA, siRNA, RNP, proteins, peptides, oligos, and small molecules.

Key Factors to Ensure a Successful Boost:

Critical Boosting Parameters



Cell Health
Happy cells yield better viability and delivery performance

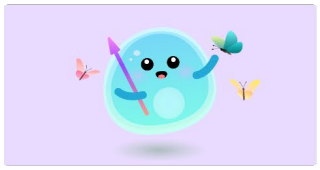
Cargo Concentration
Boosting is diffusion-based. Higher cargo concentration = higher delivery

Cell Speed + Pore Size
Smaller pore sizes and faster speeds drive cargo delivery

Cell Recovery
Membranes reseal in 30 seconds. Transfer to media and incubator

Becoming a Boost Master

High quality cell preparation and handling ensures cell health - optimize your workflow to reduce handling time!



Novice: My Boost Works (1 Wk)

- Some pore size and pressure optimization
- Standard culture conditions and cell handling
- Using standard delivery conditions

Apprentice: Good Data Often (3-5 Wks)

- Comfortable with workflow, quick cell handling
- Conscious of cell culture conditions before & after boosting
- Started to optimize secondary parameters (cell concentration, buffer, cargo source)

Master: Hot Boost Every Time (2-3 Mos)

- Optimized a routine workflow
- Optimized cargo sourcing (high quality supplier) and handling (good freeze / thaw)
- Quick experiments, consistent cell handling and culture conditions before / after boosting

2. Equipment

Gateway™

Portal's pressure regulator. At the size of a tissue box, it fits in a BSC, and the only requirement is an outlet for power. Capable of achieving pressures of 3 - 15 psi.

MicroBooster™

Portal's Boost cartridge which houses an interior core that contains pores of a specific size. Scaled appropriately for research volumes and cell concentrations, and available in pore sizes from 4 - 15 µm.

3. Materials

3.1 Supplied Materials

Kit
Gateway™ system
Metal MicroBooster holder
11 µm MicroBooster™ 050351133-01
O-rings
Forceps

Note: Instructions for disassembly and cleaning of metal MicroBooster holders can be found in Appendix 2.

3.2 Required Materials Not Supplied

Material	Portal's Preferred Product[3]
Delivery buffer	RoosterGEM™ (RoosterBio M40200)
Delivery tracer	Fluorescent dextran[4] (Thermo, various)
Cargo	E.g., protein, peptide, RNA, antibody, small molecule, RNP
Tubes	1.5 mL, 15 mL, 50 mL
Plates	CellBIND culture plates in appropriate size (Corning)
Cell strainer	70 µm mesh strainer (Falcon 352350)
Cell vial	RoosterVial™-hBM-1M-XF (RoosterBio MSC-031), RoosterVial-hAD-1M-XF (RoosterBio C46001AD), or RoosterVial-hUC-1M-XF (RoosterBio C43001UC)
Media	
MSC Culturing media	RoosterNourish™-MSC-XF (RoosterBio KT-016)
Dissociation Reagent	TrypLE Select (Thermo, 12563029)

[3] The specifically identified reagents are used and recommended by Portal. Other reagents can be used, but the protocol should be optimized to ensure high performance.

[4] Portal recommends co-delivering dextran in all samples as a delivery tracer. Portal's dextran of choice is 3 kDa Cascade Blue (Thermo Fisher D7132).

3.3 Required Equipment

Equipment	Note
Cell counter	Should provide cell diameter
Mini centrifuge	For spinning 1.5 mL tubes
Benchtop centrifuge	For spinning 15 and 50 mL conical tubes
Sonicator bath	For cleaning the metal MicroBooster Holder
Incubator	37°C & 5% CO_2
Biosafety Cabinet	

3.4 Optional Equipment for Downstream Readouts

Equipment	Note
Flow cytometer	To analyze cargo delivery and/or cell viability
Fluorescence microscope	To analyze cargo delivery

4. Cell Boosting Specifications

Parameter	Minimum	Maximum	Recommended
Pore size	10 µm	12 µm	11 µm
Volume	50 µl	200 µl	100 µl
Pressure	3 psi	15 psi	10-12 psi
Cell concentration	5 x 10^6 cells/mL	5 x 10^7 cells/mL	0.5-2 x 10^7 cells/mL
Cargo volume			≤ 10% total volume[5]
Sample number per booster	1	10	5

[5] It is not recommended to dilute the delivery buffer with the cargo buffer at a rate higher than 10% of the reaction volume, as this may alter Boost performance. If the cargo volume must exceed 10%, please adjust according to Appendix 3.

5. Protocol

5.1 Pre-Boost Cell Preparation

1. Prepare media according to Appendix 4.
2. Thaw cells according to the thawing protocol outlined in Appendix 5. Briefly, thaw a vial of cells in a 37°C water bath, then transfer cells to a 50 mL centrifuge tube containing prepared medium and mix cell suspension well. Spin to collect, and seed at an appropriate density as outlined in Appendix 5.
3. Expand and maintain cells according to the expansion options listed in Appendix 6. Microscopically monitor cell confluency starting on day 3 of culture. For optimal cell viability after boosting, do not dissociate prior to day 3.
4. When culture is >80% confluent, cells are ready to harvest for boosting.
    - For best expansion and functional performance, it is recommended to passage the cultures before reaching 90% confluence.

5.2 Boost Preparation

5.2.1 MicroBooster Preparation (Metal Holders)

1. Place the Gateway into a Biosafety cabinet (BSC), plug in the instrument, and turn the switch to the "ON" position. The purple "Boost" button will illuminate when the instrument is ready.
2. Spray the metal MicroBooster holder with 70% Ethanol. Transfer to the BSC and allow it to air dry before use. Prior to assembly, blow out any remaining ethanol with compressed air from the Gateway to ensure the holder is fully dry.
    - Ensure the O-ring is firmly in place in the top of the metal holder.
3. Use the forceps to carefully place the MicroBooster chip on the bottom half of the booster with the flat side facing up. Ensure it lies flat.

4. Screw the top of the metal booster into place. Be careful not to over-torque.
5. See Appendix 2 for metal booster disassembly and cleaning protocol following use

5.2.2 Recommended Cargo Concentration[6]

Cargo type	Recommended final concentration (1X)
Dextran	0.1 mg/mL
mRNA	0.1 mg/mL
siRNA	10 µM
Peptides	50 µM
CRISPR RNP[7]	Nuclease: 0.2 mg/mL, complex with guide at a 2.5:1 guide:nuclease molar ratio
Plasmid DNA[8]	0.2 mg/mL

[6] This list represents some commonly delivered cargo. For delivery of unlisted cargo types, Portal recommends 0.1 mg/ml as a starting concentration.

[7] See Appendix 8 for Portal's RNP complexing protocol.

[8] Boosting delivers cargo to the cytosol, but in some cases expression can be seen when DNA is able to traffic to the nucleus. Results may vary by cell type and plasmid design.

5.2.3 Cargo Preparation

1. Ensure cargo is at an appropriate temperature (e.g., dextran can be kept at room temperature, while RNA should be maintained on ice until ready to boost).
    - Ensure the temperature of the cells does not drop when using cargo on ice.
    - Cells and cargo may be prepared simultaneously in order to maintain a time-efficient workflow, however, the experimental flow should ensure that cells are handled out of the incubator for as short a time as possible to maintain cell health (<30 min).
2. Prepare cargo at a 2X concentration in the delivery buffer to be added to a 2X cell solution to make 1X final for both cells and cargo when mixed.
    - Wait to add the cargo to cells until immediately prior to boosting.
    - For samples where the cargo comprises ≥10% the total reaction volume, the osmotic concentration should be rebalanced by using a 10X PBS or NaCl solution as described in Appendix 3.
    - Include an extra cargo reaction for a "no boost" control
    - When preparing the same cargo for multiple samples, make at least 10% more to allow overage.

5.2.4 Cargo Table

Experimental Group	Cargo	Stock Conc. (mg/ml)	Boost Conc. (2X) (µg/ml)	Total µg Needed	Vol of Stock (µL)	Volume of Buffer (µL)	Total Cargo Vol (µL)
Untreated/No cargo control	-	-	-	-	-	50	50
For one reaction	Cargo 1						
	Cargo 2						
For all cargo conditions (including "No Boost" control)	Cargo 1						
	Cargo 2						

5.3 Cell Preparation

1. Cells for boost should be cultured in appropriate T75 or T225 flasks. Cells should be passaged at least 72 hours before boosting and should be around 70-80% confluence when harvested.
2. For harvest, transfer flasks into the biosafety cabinet and remove media.
3. Add 3 mL TrypLE to each T75 flask or 10 mL TrypLE to each T225 flask and place flasks in 37°C (5% CO_2) incubator to dissociate cells.
    - Check culture every 5 min until cells are detached from the surface.
4. Add an equivalent volume of RoosterNourish-MSC-XF to each vessel to stop the TrypLE activity.
5. Transfer the cell suspension into a 50 mL centrifuge tube.
6. Centrifuge at 350 x g for 5 minutes.
7. Aspirate the supernatant and resuspend cells in 5 mL (T75) or 15 mL (T225) RoosterNourish-MSC-XF media.
8. Pass cells through a 70 µm mesh strainer to remove large aggregate or debris.
9. Count cells with a cell counting device and note cell diameter.

Sample	Concentration	Viability	Volume	Total Live Cells	Volume to Resuspend	Cell Diameter

10. Centrifuge at 350 x g for 5 minutes.
11. Resuspend cells in RoosterGEM at 2X concentration for a final 1X concentration within the range of 5 x 10^6 - 1 x 10^7 cells/mL.

5.4 Cell Boost

1. Prepare a 1.5 mL tube for collection of each sample post-boost. Prepare additional tubes for mixing samples pre-boost and collecting wash buffer between boosts.
2. Prepare **untreated control** sample by mixing equal volumes of 2X cells and delivery buffer in a 1.5 mL tube.
3. Prepare **"no boost" control** sample by mixing equal volumes of 2X cargo with 2X cells in a 1.5 mL tube.
4. Before boosting cells, wash the MicroBooster with 100 µL delivery buffer at desired boost pressure[9] as follows:
    a. Set the pressure on the Gateway by turning the purple knob until the pressure gauge indicates the appropriate pressure. Wait until the purple indicator light is ready. Press and hold the purple button until the light turns off to empty the tank. Wait for the pressure tank to refill and the indicator light to turn back on[10].
        - Emptying the tank is an important step to ensure sufficient and consistent pressure is available for the duration of the boost. The tank should be emptied each time a new pressure is set.
    b. Place a collection tube in the holder on the Gateway and secure the cap by placing it on the side of the holder.
    c. Pipette 100 µL of delivery buffer into the top of the MicroBooster.
    d. Connect the MicroBooster to the Gateway by screwing into the luer lock on top until secure. Be careful not to over-torque.
    e. Raise the collection tube below the booster.
    f. Press and hold the purple button to pressurize the system until the indicator light turns off. The delivery buffer should flow through the booster.
    g. Discard the delivery buffer.
5. For each boost condition, mix equal volumes of 2X cells with 2X cargo (or an equal volume of delivery buffer for a "no cargo" control, if desired). For optimal performance, only mix cells and cargo immediately prior[11] to loading the MicroBooster.
    a. Place a collection tube in the holder on the Gateway and secure the cap by placing it on the side of the holder.
    b. Transfer the cells + cargo solution into the top of the MicroBooster.
    c. Set the desired pressure on the Gateway by turning the purple knob until the pressure gauge indicates the appropriate pressure. Wait until the purple indicator light is ready. When changing pressure, press and hold the purple button until the light turns off, and wait for the pressure tank to refill and indicator light to turn back on before proceeding with boost.
        - Emptying the tank is an important step to ensure sufficient and consistent pressure is available for the duration of the boost. The tank should be emptied each time a new pressure is set.
    d. Connect the MicroBooster to the Gateway and lift the collection tube into place below the MicroBooster.

e. Boost the solution into the collection tube by pressing the button until the indicator light turns off.
   - If the indicator light turns off prior to boost completion, the tank has de-pressurized. Release the button and allow the tank to re-pressurize and the light to turn back on before the boost can be continued, if necessary.

f. Remove the MicroBooster from the Gateway by unscrewing and remove the collection tube from the holder.

g. Allow cell membranes to close for at least 30 seconds.

h. Add 1 mL RoosterNourish to the 1.5 mL tube[12].

6. To prepare the MicroBooster for the next sample, wait at least 30 seconds after the boost and repeat step 4 to wash the booster with 100 µL of delivery buffer. Collect in a fresh 1.5 mL tube to discard.
7. Repeat steps 5-6 for each sample.
   a. The same booster can be used for multiple boosts within a single experiment, provided that the cell type and cargo type are the same. Portal recommends using a new booster after 5 boosts, although variables including the 'stickiness' of cells may impact this number.
8. When all samples have been collected, move to cell processing for downstream applications and/or analysis[13]. This may include but is not limited to preparing cells for culturing, flow cytometry analysis, or microscopy.
9. For same day readout, transfer 200 µL to a 96-well V-bottom plate for flow cytometry (see Appendix 7 for flow cytometry protocol).
10. To culture cells overnight, spin the 1.5 mL tubes at 350 x g for 5 minutes at RT.
11. Resuspend cells in 1 mL complete media.
12. Transfer cells into a 6 well plate in prepared RoosterNourish media for overnight culturing in 37°C incubator.
    a. Cells should be cultured at 3,000-30,000 cells/cm^2 at 37°C. Cells can be counted to adjust concentration.

[9] See Boosting Specifications table for recommended pressure for optimal performance in MSCs. A pressure sweep may be performed to optimize performance for specific use cases.

[10] If the pressure does not return to the initially set pressure, reset the gauge and test again. This should ensure that the gauge is at the right pressure for boosting. Check the gauge periodically throughout the experiment, and periodic pressure resetting may be required. A pressure difference of 1-2 psi is not thought to have a significant effect on performance.

[11] Delaying mixing until immediately prior to Cell Boost is most important for sensitive cargo (i.e., RNA).

[12] Solution may splash on the side of the 1.5 mL tube while boosting. RoosterNourish may be used to wash boosted sample from the side of the tube; avoid spinning the tube until after RoosterNourish is added.

[13] If fluorescent dextran is used as a delivery tracer, cells should be washed at least once prior to downstream analysis and/or culturing to minimize background signal and cellular uptake through endocytosis in culture.

6. Sample data

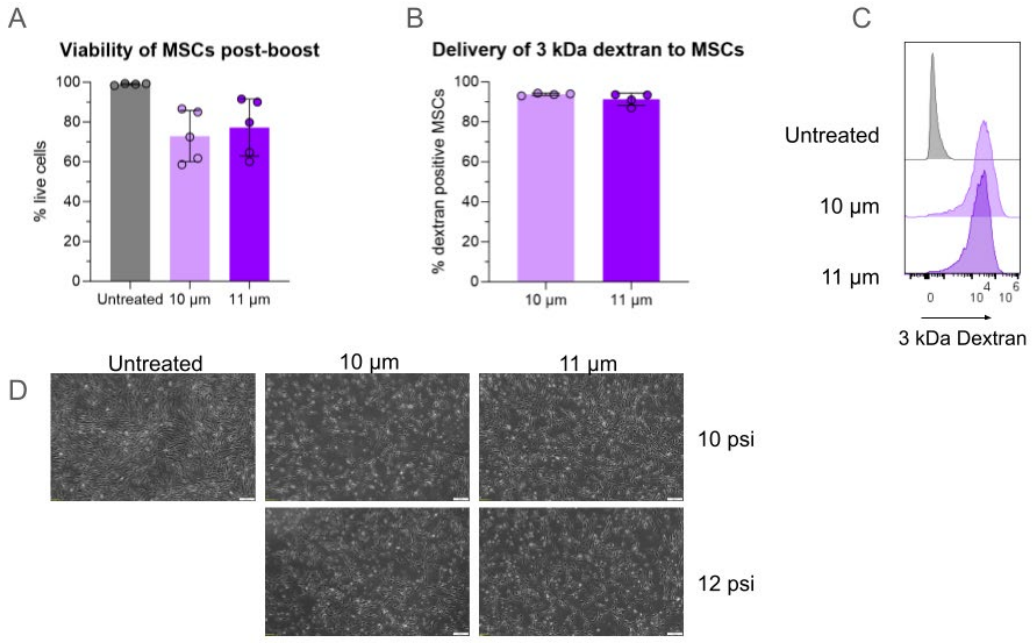


Figure 1. Mesenchymal Stem Cells (MSCs) tolerate boosting well with efficient delivery. Representative viability (A) and dextran delivery (B, C) of MSCs following boost using 10 and 11 µm pore sizes at 11 psi, N=2 experiments. Viability and delivery were measured via flow cytometry. (D) Images of MSCs taken 24 hrs post-boost show maintenance of cell morphology following boost. Scale bar = 200 µm.

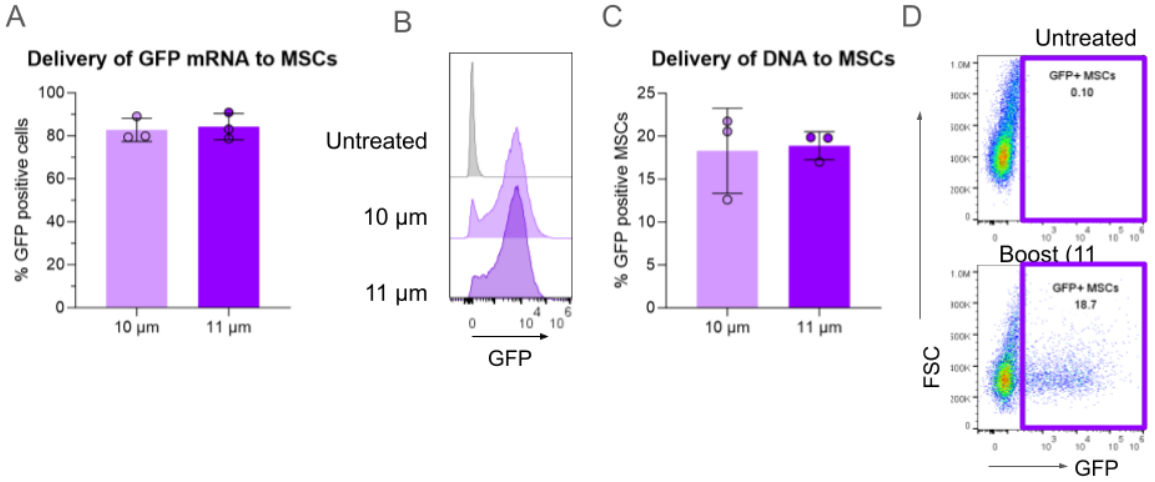


Figure 2. Efficient delivery and performance of biological cargoes observed in MSCs. Representative expression of GFP following delivery of GFP mRNA (A, B) or GFP plasmid (C, D) in MSCs, using 10 and 11 µm pore sizes at 11 psi. Delivery was measured using flow cytometry, N=2 experiments.

Appendix 1 - Notes and Tips

- Call us! We're happy to help.
  - For boosting guidance (info@portal.bio)
  - For MSC culture guidance (info@roosterbio.com)
- Results may be donor-dependent.
- For optimal cell performance, experiments should be concise and completed in a short timeframe (ideally <30 minutes) to reduce cell stress.
- It is ideal to add sensitive cargo (e.g., mRNA) to cells immediately prior to boosting for optimal performance.
- Temperature can affect performance. Lower temperatures are more harsh on cells but can increase delivery. Pre-warming all media and buffers can help to preserve cell health.
- At research scale, Portal has found cell concentration has little to no impact on performance; however cargo concentration can impact delivery performance and when in doubt a cargo titration should be performed. When cargo is in excess (in the recommended ranges), there is no need to change the cargo concentration when cell concentration changes, or when multiplexing cargos.

Appendix 2 - Metal Booster Cleaning

1. Disassemble metal holder and discard the chip with forceps.
2. Spray metal holder with 70% Ethanol.
3. Place in a clean container with DI water and sonicate for 10 minutes in a sonicator bath.
4. Spray metal holders with 70% Ethanol and let air dry.

Appendix 3 - Adjusting Osmolarity

Cargo concentration should be <10% of total reaction volume (i.e. <10 µL for a 100 µL total volume). If cargo is $\geq$ 10% of reaction volume, use 10X PBS or NaCl to preserve osmolarity.

- For use of 10X PBS: add an appropriate volume of 10X PBS to cargo solution such that the cargo volume is at 1X salt solution
  - i.e. if the cargo added is 20 µl, add 2.22 µl 10X PBS for a final volume of 22.2 at 1X PBS concentration
- For use of NaCl: add an appropriate volume of 5 M NaCl to the cargo solution such that the cargo volume is at 0.154 M or 0.9% NaCl by mass.
  - i.e. if the cargo added is 20 µl, add 0.6 µl of 5M NaCl

Appendix 4 - Media Preparation

1. Bring RoosterNourish™-MSC-XF components to room temperature, protected from light, for up to four hours.
2. Prepare 1 bottle of medium by aseptically adding 1 bottle of RoosterBooster™-MSC-XF (Part No. SU-016) to 1 bottle of RoosterBasal™-MSC (Part No. SU-005/SU-022/ M22520).
3. Mix well by capping and gently mixing the bottle.

Appendix 5 - Cell Thawing & Seeding

1. Aseptically transfer 10 mL of RoosterNourish-MSC-XF into a 50 mL centrifuge tube.
2. Thaw RoosterVial-hBM-1M-XF (or equivalent adipose MSC or umbilical cord MSC) vial in an automated thawing device (e.g., ThawStar), or manually in a 37°C water bath. When thawing in a water bath, monitor the vial closely and remove from water bath once only a small bit of ice is remaining (2-3 minutes).
3. Aseptically transfer vial into a Biosafety Cabinet (BSC).
4. Transfer vial contents into the 50 mL centrifuge tube containing prepared medium and mix cell suspension well.
5. Centrifuge at 350 x g for 10 min.
6. Aspirate the supernatant and resuspend cells in 20 mL of RoosterNourish-MSC-XF medium.
7. Mix well and seed cells equally into four T75 vessels or into two T225 vessels, and add medium to bring volume up to final volume according to table below:

Type of culture vessel	Total volume of cell suspension per vessel	Final volume per vessel
T75 x 4	5 mL	15 mL
T225 X 2	10 mL	45 mL

8. Transfer vessels into an incubator (37°C, 5% CO2) and ensure surfaces are covered evenly with media.
9. Microscopically monitor cell confluency starting on day 3 of culture. For optimal cell viability after boosting, do not dissociate prior to day 3.
10. When culture is >80% confluent, cells are ready to harvest.
    - For best expansion and functional performance, it is recommended to passage the cultures before reaching 90% confluence. If the cultures reach over confluence, this may result in difficulty when harvesting, increased aggregation, decreased cell viability, growth inhibition and loss of differentiation potential.

Appendix 6 - MSC Expansion Options

Please refer to the following Expansion Options table to determine the cell culture vessel best suited to your research needs. Note: If larger cell numbers are required, a decrease in seeding density to 2,200 cells/cm^2 (and increase in total vessel seeding surface area to 450 cm^2 and total seeding media to 90 mL) will maximize total cell output at harvest with an increase in total Population Doubling Level (PDL).

Vessel	Surface Area (cm^2)	Number of Vessels Needed	Total surface Area (cm2)	Seeding Density (Cells/cm^2)	Approx. Yield at Harvest	Days of Culture
T75	75	4	300	3,300	≥ 10M	3 to 6
T225	225	2	450	2,200	≥ 12M	3 to 6

Appendix 7 - Flow Cytometry Analysis

Appendix 7.1 Required Materials Not Supplied

Material	Portal's Preferred Product[13]	Recipe Amount
FACS buffer	Phosphate Buffered Saline (PBS), pH 7.4 (Gibco 10010072)	
	Heat-inactivated Fetal Bovine Serum (HI-FBS)	2%
	EDTA, 0.5M pH 8.0 (Invitrogen AM9261)	0.2%
Viability stain	Live/Dead Fixable Near IR Cell Stain (Thermo L10119)	
Plate	96-well V-bottom (for use with autosampler)	

[13] The specifically identified reagents are used and recommended by Portal. Other reagents can be used, but the protocol should be optimized to ensure high performance.

Appendix 7.2 Flow Cytometry Protocol

1. Transfer 200 µL of cells in complete media to a 96-well V-bottom plate for flow cytometry.
2. Centrifuge plate at 350 x g, 6 minutes, discard supernatant by flicking plate.
3. Resuspend cells in 200 µL PBS, centrifuge plate at 350 x g, 6 minutes, discard supernatant by flicking plate.
4. For viability stain, resuspend cells in 100 µL of 1:1000 solution of Live/Dead Fixable Cell Stain in PBS.
5. Incubate covered for 30 minutes at RT.
6. For antibody staining, dilute to appropriate concentration as indicated by the manufacturer.
7. Add 100 µL FACS buffer to dilute the viability stain and/or antibodies and wash.
8. Centrifuge plate at 350 x g, 6 minutes, discard supernatant by flicking plate.
9. Resuspend in 200 µL FACS buffer and repeat step 6 to wash.
10. Resuspend in 200 µL FACS buffer and proceed immediately to flow cytometry.

If necessary, store plate in the dark at 4°C until ready for flow cytometry.

Appendix 8 - RNP Workflow

Appendix 8.1 Required Materials Not Supplied

Material	Portal's Preferred Product[14]	Final concentration
Cas9 nuclease	IDT (10007808)	0.2 mg/ml (~1.27 pmol/µl)
sgRNA	IDT or Synthego	0.1 mg/ml (~3.15 pmol/µl)

[14]The specifically identified reagents are used and recommended by Portal. Other reagents can be used, but the protocol should be optimized to ensure high performance.

Appendix 8.2 RNP Pre-complexing Protocol

1. Thaw sgRNA on ice.
2. Pipette the required amount of Cas9 into a 1.5 ml tube.
3. Add the required amount of sgRNA to the Cas9 and mix well.
    - Portal recommends using a 1:2.5 molar ratio of Cas9:sgRNA.
    - When pooling multiple guides, Portal recommends using the recommended amount per guide.
4. Incubate at RT for 15 minutes.
    - If not ready to use the RNP immediately, return to ice until ready to add cargo to cells.
5. Prior to boosting, add delivery buffer and any additional cargo components to complete the 2X cargo mixture.
    - I.e. for a 100 µl boost (cells + cargo), prepare a 50 µl cargo solution containing 20 µg (~127 pmol) of Cas9 and 10 µg (~315 pmol) of sgRNA.
    - If cargo is ≥10% of total reaction volume, see Appendix 3 to adjust osmolarity.