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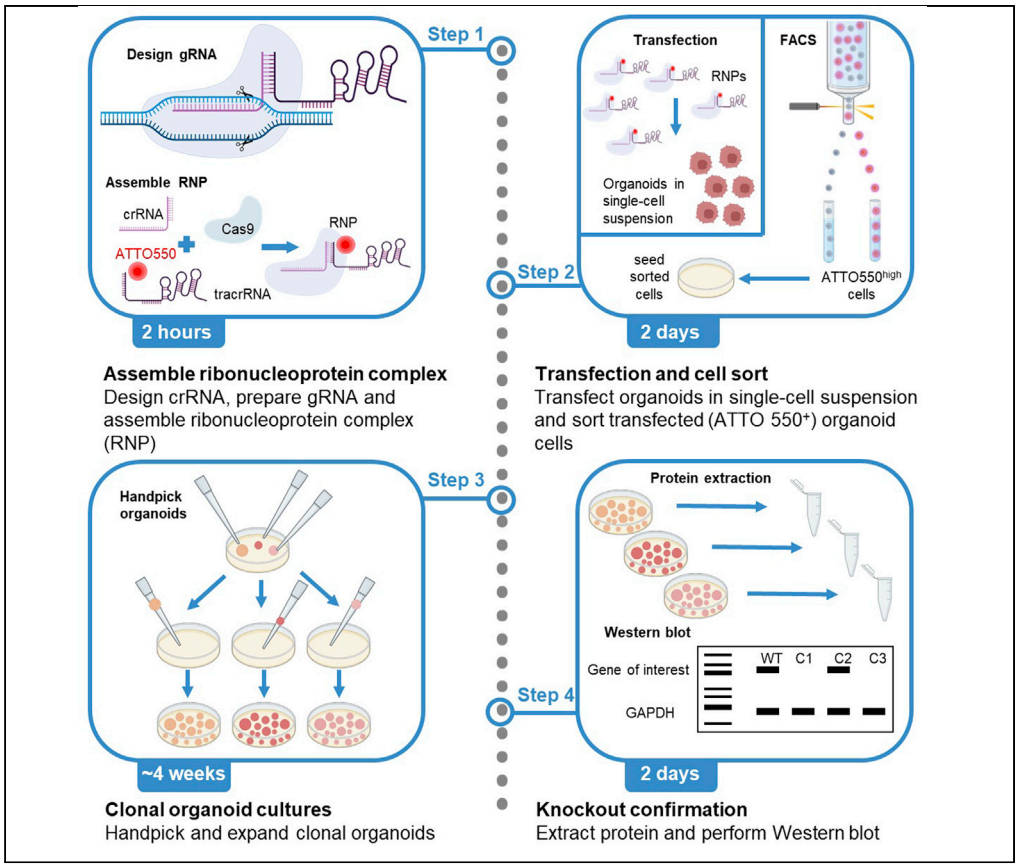
Generation of gene-of-interest knockouts in murine organoids using CRISPR-Cas9



Gene-of-interest knockout organoids present a powerful and versatile research tool to study a gene's effects on many biological and pathological processes. Here, we present a straightforward and broadly applicable protocol to generate gene knockouts in mouse organoids using CRISPR-Cas9 technology. We describe the processes of transient transfecting organoids with pre-assembled CRISPR-Cas9 ribonucleoprotein complexes, organoid cell sorting, and establishing clonal organoid culture pairs. We then detail how to confirm the knockout via Western blot analysis.

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

Anne Huber,
Christine Dijkstra,
Matthias Ernst,
Moritz F. Eissmann

anne.huber@onjcri.org.au (A.H.)
moritz.eismann@onjcri.org.au (M.F.E.)

Highlights

Step-by-step protocol to generate clonal gene knockout/parental organoid culture pairs

Gene editing technique designed to minimize genetic and phenotypic off-target effects

Gene knockout organoids are versatile tools for many biological research questions

Broadly accessible protocol due to no prior cloning and reduced equipment requirements

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Generation of gene-of-interest knockouts in murine organoids using CRISPR-Cas9

Anne Huber,[1,2,*] Christine Dijkstra,[1] Matthias Ernst,[1] and Moritz F. Eissmann[1,3,*]

[1]Olivia Newton-John Cancer Research Institute and La Trobe School of Cancer Medicine, La Trobe University, Melbourne, VIC 3084, Australia

[2]Technical contact

[3]Lead contact

*Correspondence: anne.huber@onjcri.org.au (A.H.), moritz.eismann@onjcri.org.au (M.F.E.)
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SUMMARY

Gene-of-interest knockout organoids present a powerful and versatile research tool to study a gene's effects on many biological and pathological processes. Here, we present a straightforward and broadly applicable protocol to generate gene knockouts in mouse organoids using CRISPR-Cas9 technology. We describe the processes of transient transfecting organoids with pre-assembled CRISPR-Cas9 ribonucleoprotein complexes, organoid cell sorting, and establishing clonal organoid culture pairs. We then detail how to confirm the knockout via Western blot analysis.

BEFORE YOU BEGIN

The protocol below describes the specific steps to generate a gene knockout in murine gastric cancer organoids using the Alt-R™ CRISPR-Cas9 system from Integrated DNA Technologies (IDT). This system uses transient transfection of ribonucleoprotein complex (RNP) that consists of a guide RNA (gRNA) and the wild-type *S. pyogenes* Cas9 nuclease.

We have successfully employed the described protocol to generate knockouts of four different target genes in two independent murine gastric cancer (GC) organoid cultures; the first are antrum tumor organoids established from *gp130*$^{Y757F/Y757F}$ mutant mice[1] and the second was derived from *gp130* wild-type murine gastric carcinoma mice (developed by MFE, *manuscript in preparation*). While, we have employed this protocol for murine gastric cancer organoids, we believe the method of gene editing described here is versatile and applicable to a broad range of murine or human organoids. For example, similar methodology has been used to gene edit primary human liver,[2,3] colon,[4] intestinal[5,6] or fetal lung[7] and murine colon[8] or intestinal organoids.[9] We predict any organoid culture that can be transfected with Lipofectamine and that displays robust growth can be used for this protocol. Growth media and conditions will need to be optimized for each organoid model.

Institutional permissions

All murine tissues and samples used for these experiments have been obtained in accordance with all relevant ethical regulations for animal testing and research, including the Australian code for the care and use of animals for scientific purposes, and were approved by the Animal Ethics Committee of Austin Health and La Trobe University, Australia. Genetic manipulations were in accordance and under the approval of the Institutional Biosafety Committee of the Olivia Newton-John Cancer Research Institute.

Designing and ordering guide RNAs

Timing: 1 h

Note: We used the Alt-R™ CRISPR-Cas9 system from IDT. The gRNA is composed of a sequence-specific CRISPR RNA (crRNA) and a transactivating crRNA (tracrRNA). You can either order predesigned crRNAs (https://sg.idtdna.com/site/order/designtool/index/CRISPR_PREDESIGN) or use a published sequence as a template to design a crRNA (https://sg.idtdna.com/site/order/designtool/index/CRISPR_ SEQUENCE). The 67nt tracrRNA of the Alt-R™ system is shorter than the natural *S. pyogenes* tracrRNA that contains 89 nt and is fluorescently labeled with ATTO™ 550. The fluorescent dye allows the monitoring of transfection efficiency and sorting of successfully transfected cells.

Note: For more information and recommendations on CRISPR gRNA design, please have a look at these comprehensive reviews by Mohr et al. and Wilson et al.[10,11]

1. Search for predesigned crRNAs or check your own crRNA design via the online tool on the IDT website.
2. Order the selected crRNAs and ATTO™ 550 tracrRNAs.

Note: When selecting the predesigned crRNAs from IDT or checking your own crRNAs on the IDT website, the results include helpful details such as the On- and Off-target score which indicate the predicted editing performance and off-target risks.

Note: We recommend selecting multiple crRNAs per target gene to not only serve as backups, but more importantly, establishing a gene knockout with different gRNAs will help to exclude off-target editing effects in downstream analysis.

Preparation of organoid medium

Timing: 15 min

Note: We used IntestiCult™ Organoid Growth Medium (Mouse) from STEMCELL Technologies. Alternative growth media can be used. Optimization of growth conditions is required when using other organ-derived organoids and/or cell lines.

3. Supplement IntestiCult™ OGM Mouse Basal medium (90 mL) with 5 mL of IntestiCult™ OGM Mouse Supplement 1 and 5 mL of IntestiCult™ OGM Mouse Supplement 2.
4. Store complete medium at −20°C for up to 3 months.
5. Add Penicillin/Streptomycin (1:100 dilution; stock solution 10,000 units/mL Penicillin and 10,000 µg/mL Streptomycin) before use. After thawing and adding Penicillin/Streptomycin IntestiCult™ can be stored at 4°C for up to 5 days.

Murine gastric cancer organoid culture

Timing: 3 weeks

Note: In this protocol, we describe the use of Cultrex Reduced Growth Factor Basement Membrane Extract, Type 2, Pathclear for growing organoids. However, in our experience, Matrigel can be used interchangeably if the organoids grow efficiently. Similarly, we describe the use of IntestiCult™ Organoid Growth Medium for the culture of our GC organoids, although any other media in which the organoids of choice are known to grow, might be used alternatively.

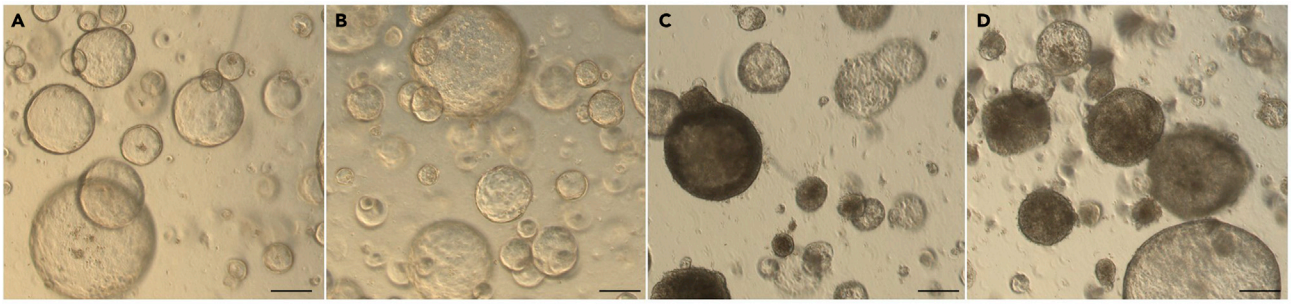

Figure 1. Examples of murine gastric cancer organoid cultures

(A and B) Show healthy murine gastric cancer organoid cultures in growth phase. Optimal phase to passage organoids or employ organoids in transfection.

(C and D) Show murine gastric cancer organoid cultures in death phase. Do not use organoid in death phase to passage or transfect. Scalebar represent 200 μm.

Note: Use organoids in the earliest passage possible to limit the accumulation of spontaneous mutations and phenotypical changes before gene editing is conducted. Passage the organoids at least twice after thawing to ensure optimal health and proliferation rate of the organoid culture before commencing the CRISPR-Cas9 editing procedures.

6. Thaw a vial of cryopreserved murine organoids and transfer into 5 mL of Advanced DMEM/F-12 supplemented with 10% Fetal Bovine Serum and Penicillin/Streptomycin (1:100 dilution) in a 10 mL tube.
7. Centrifuge at 4°C at 300 × *g* for 5 min and remove supernatant.
8. Resuspend organoids in 1 mL of Advanced DMEM/F-12 supplemented with with 10% Fetal Bovine Serum and Penicillin/Streptomycin (1:100 dilution) and transfer to a 1.5 mL Eppendorf tube.
9. Centrifuge at 4°C at 300 × *g* for 5 min and remove supernatant.
10. Resuspend organoids in Cultrex Reduced Growth Factor Basement Membrane Extract, Type 2, Pathclear (RGF BME) and pipette 50 μL into each well of a pre-warmed 24-well plate. Typically, 500 cryopreserved organoids are resuspended in 150 μL RGF BME.
11. Place the plate in a 37°C. 10% CO_2 incubator for 10 min to solidify the RGF BME.
12. Add 500 μL of IntestiCult™ Organoid Growth Medium supplemented with Penicillin/Streptomycin (1:100 dilution) into each well.
13. Change the medium every 3–5 days.

Note: Growth kinetics can vary depending on the nature of the organoids used. Our murine GC organoids recover their morphology 6–8 days after thawing. Healthy organoids subsequently increase their size. Typically, murine GC organoids are expanded every 5–7 days after thawing. Only passage organoids when they have reached growth phase (Figures 1A and 1B).

14. To passage organoids, collect each well of organoids in 1 mL of Gentle Cell Dissociation Reagent (STEMCELL Technologies) and transfer to a 10 mL tube.
15. Incubate at 20°C–25°C for 10 min on orbital roller.
16. Centrifuge at 4°C at 300 × *g* for 5 min and remove supernatant.
17. Resuspend pellets in 10 mL of Advanced DMEM/F-12 supplemented with 10% Fetal Bovine Serum and Penicillin/Streptomycin (1:100 dilution).
18. Wash, plate and incubate the organoids as described in steps 7–12 for 5–7 days or until they reach their growth phase before next passaging. Passage the organoids at least twice after thawing to ensure optimal health and proliferation rate and then, start the transfection protocol with healthy organoids in their growth phase (see example images in Figure 1).

Troubleshooting 1: If the organoids grow slower than usual, prepare fresh medium, ensure all components are added and exchange medium on organoids every 2 days.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-GAPDH antibody, Mouse monoclonal, clone GAPDH-71.1, 1:2000 dilution	Sigma-Aldrich	Cat# G8795
IRDye 680RD Goat anti-Mouse IgG (H+L), 1:10,000 dilution	LI-COR	Cat# 926-68070
IRDye 800CW Goat anti-Rabbit IgG (H+L), 1:10,000 dilution	LI-COR	Cat# 926-32211
Biological samples		
Antrum tumor organoids (culture passage 10) derived from *female gp130$^{Y757F/Y757F}$* mutant mice (C57/BL6 background)	Established by MFE; details in Eissmann et al.[1]	Eissmann et al.[1]
gastric carcinoma organoids (culture passage 12) derived from female Kras;Pik3ca;Trp53 mutant mouse (C57/BL6 background)	established by MFE, (*manuscript in preparation*)	N/A
Chemicals, peptides, and recombinant proteins		
Advanced DMEM/F-12	Thermo Fisher Scientific	Cat# 12634010
Alt-R *Streptococcus pyogenes* Cas9 nuclease V3	Integrated DNA Technologies	Cat# 1081058
cOmplete, Mini, EDTA-free Protease Inhibitor Cocktail	Roche	Cat# 11836170001
Cultrex Reduced Growth Factor Basement Membrane Extract, Type 2, Pathclear (RGF BME)	R&D Systems	Cat# 3533-005-02
DPBS	Thermo Fisher Scientific	Cat# 14190144
Fetal bovine serum (FBS)	Bovogen biologicals	Cat# SFBS-AU
Gentle cell dissociation reagent	STEMCELL Technologies	Cat# 100-0485
Immobilon-FL PVDF membrane	Millipore	Cat# IPFL00010
Intercept (TBS) blocking buffer	LI-COR	Cat# 927-60001
IntestiCult™ Organoid Growth Medium (Mouse)	STEMCELL Technologies	Cat# 06005
Lipofectamine RNAiMAX Transfection reagent	Thermo Fisher Scientific	Cat# 13778030
Nuclease-Free Duplex Buffer	Integrated DNA Technologies	Cat# 11-01-03-01
NuPAGE 4%–12%, Bis-Tris, 1.0–1.5 mm, Mini Protein Gels	Thermo Fisher Scientific	Cat# NP0335BOX
NuPAGE LDS Sample Buffer (4×)	Thermo Fisher Scientific	Cat# NP0007
NuPage MES SDS Running Buffer (20×)	Thermo Fisher Scientific	Cat# NP000202
NuPAGE Sample Reducing Agent (10×)	Thermo Fisher Scientific	Cat# NP0004
Opti-MEM	Thermo Fisher Scientific	Cat# 31985062
Penicillin/Streptomycin	Thermo Fisher Scientific	Cat# 15140122
PhosSTOP	Roche	Cat# 4906845001
Precision plus Protein Kaleidoscope Prestained Protein Standards	Bio-Rad Laboratories	Cat# 1610375
Skim Milk Powder	Devondale	N/A
SYTOX™ Blue Nucleic Acid Stain	Thermo Fisher Scientific	Cat# S11348
TrypLE Express	Thermo Fisher Scientific	Cat# 12604021
Critical commercial assays		
Pierce™ BCA Protein Assay Kit	Thermo Fisher Scientific	Cat# 23225
Oligonucleotides		
Alt-R CRISPR-Cas9 crRNA, 2nmol	Integrated DNA Technologies	N/A
Alt-R CRISPR-Cas9 tracrRNA-ATTO™ 550	Integrated DNA Technologies	Cat# 1075928

MATERIALS AND EQUIPMENT

FACS buffer

Reagent	Final concentration	Amount
DPBS	N/A	45 mL
FBS	10%	5 mL
Total	**N/A**	**50 mL**

RIPA lysis buffer		
Reagent	Final concentration	Amount
100% Glycerol	10%	5 mL
1 M Tris pH 7.5	20 mM	1 mL
5 M NaCl	137 mM	1.37 mL
20% SDS	0.1%	0.25 mL
10% NP-40	0.5%	2.5 mL
10% Tx-100	1.0%	5 mL
0.5 M EDTA	2 mM	0.2 mL
ddH2O	N/A	34.68 mL
Total	**N/A**	**50 mL**

Transfer buffer (10×)		
Reagent	Final concentration	Amount
Glycine	192 mM	144 g
Tris base	25 mM	30.2 g
Methanol	20%	Add to 1× Transfer buffer before use
Total	**N/A**	**1 L**

TBS (10×)		
Reagent	Final concentration	Amount
Tris base	200 mM	48 g
NaCl	1,500 mM	176 g
12 N HCl	pH 7.6	N/A
ddH2O	N/A	Fill up to 2 L
Tween 20 detergent	0.1% (w/v)	Add to 1× TBS buffer before use
Total	**N/A**	**2 L**

STEP-BY-STEP METHOD DETAILS

This protocol describes the generation of gene of interest (GOI) knockouts in murine organoids utilizing the Alt-R™ CRISPR-Cas9 system from Integrated DNA Technologies (IDT). We successfully applied this protocol to generate knockouts of four different genes in two independent murine GC organoids. We describe a straightforward method to establish GOI knockout and parental organoid (or cell line) culture pairs. No prior genetic manipulations are required. All CRISPR-Cas9-editing components are only transiently transfected, which is predicted to minimize the risk of phenotypic changes arising from "off-target" editing, persistent expression of CRISPR-Cas9 constructs or presence of viral elements.[12–17] Therefore, generated parental and KO organoid cultures will be ideal tools to address a broad range of biological questions such as GOI's involvement in stemness, carcinogenesis, therapy responses, cell-cell interactions, epithelial/immune cell, and stromal/immune cell cross talk. Similar methodology, particularly transient transfection or nucleofection of the RNA editing complex and the CRISPR-Cas9 enzyme for gene editing in organoids have been described.[2–7] However, these protocols introduce additional plasmids or gene knock-ins, which may not be desired for some downstream applications i.e., knock-in of transgene encoding a fluorescence protein may increase immunogenicity of the organoids in *in vivo* tumor models.[18–21] Therefore, the straightforward step-by step protocol described here, is ideally suited to generate gene knockout organoids with a focus on minimally altering the genotype and phenotype of the original organoid cultures.

Preparation of gRNA and forming of the RNP complex

Timing: 1 h

This step outlines the resuspension of the RNA oligos, the preparation of the gRNA and finally the formation of the RNP complex.

1. Resuspend each Alt-R CRISPR-Cas9 RNA oligo from IDT in Nuclease-Free Duplex Buffer to 100 μM stock concentration. If you purchase the RNA oligos at 2 nmol, resuspend in 20 μL.

   Pause point: You can store the stock at −20°C.

2. Mix the crRNA and tracrRNA so that the final duplex has a concentration of 1 μM.

Reagent	Amount
100 μM crRNA	1 μL
100 μM tracrRNA	1 μL
Nuclease-Free Duplex Buffer	98 μL
Total	100 μL

3. Heat at 95°C for 5 min.
4. Allow to cool down to 20°C–25°C.

   Pause point: You can store the crRNA:tracrRNA duplexes for at least 6 months at −20°C.

5. Dilute Alt-R *Streptococcus pyogenes* Cas9 nuclease to 1 μM in Opti-MEM.
6. Combine the following to assemble the RNP complex. The amount given is for one well of a 48-well plate used for transfection.

Reagent	Amount
1 μM gRNA	3 μL
1 μM Cas9 nuclease	3 μL
Opti-MEM	44 μL
Total	50 μL

7. Incubate at 20°C–25°C for 5 min.

   Pause point: You can store the assembled RNP complex for up to 4 weeks at 4°C or for up to 6 months at −80°C.

Preparation of organoids for transfection

Timing: 1 h

Note: Timing depends on the number of organoids required for the transfection. One hour refers to the preparation of 5 wells and timing increases by approximately 10 min for each additional 5 wells included at the start.

This step describes the preparation of a (nearly) single-cell suspension of murine organoids using trypsin in preparation for the transfection of the CRISPR-Cas9 editing components (Figure 2). Start with healthy organoids in their growth phase (examples in Figure 1).

8. Pre-warm TrypLE and Advanced DMEM/F-12 supplemented with 10% Fetal Bovine Serum and Penicillin/Streptomycin (1:100 dilution) in 37°C water bath.

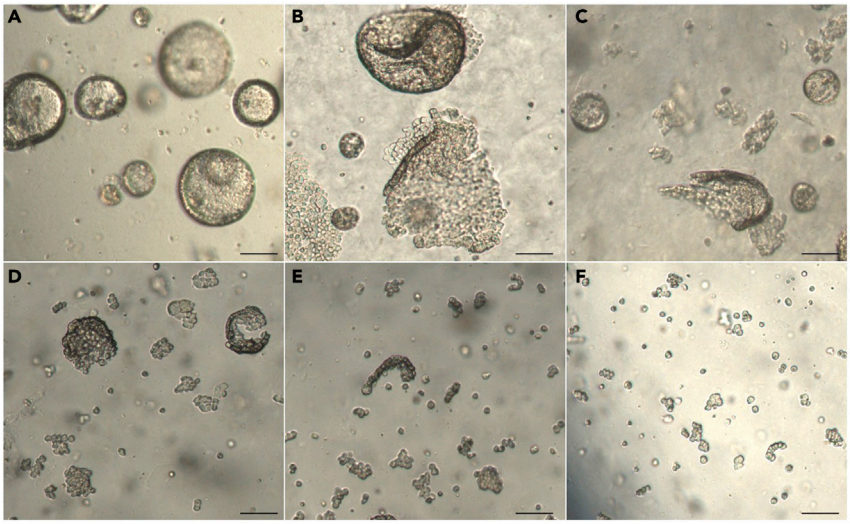

Figure 2. Preparing single cells from organoids using TrypLE solution

(A) Shows healthy murine gastric cancer organoids in growth phase, the optimal phase to start preparation of organoids for transfection.

(B) Shows partially broken up organoids in PBS after transferring to 15 mL tube (step 10).

(C) Shows partially broken up organoids after resuspension in pre-warmed TrypLE solution (step 13).

(D) Shows dissociating organoids after incubation in TrypLE solution at 37°C for 5 min (step 15).

(E) Shows dissociating organoids after incubation in TrypLE solution at 37°C for a total of 10 min (step 17).

(F) Shows small clumps of cells or single cells from dissociated organoids after incubation in TrypLE solution for a total of 15 min (step 17). Scalebar represent 100 μm.

9. Replace the organoid medium in each well with 500 μL of cold PBS.
10. Transfer organoids from all wells (500 organoids required per well of the 48-well plate for transfection) into 15 mL tube. Each well should then be rinsed again with 500 μL of cold PBS to collect possible remnants of RGF BME with organoids that might still be in the wells; this should be transferred to the same tube.
11. Place tube on ice for 5 min.
12. Centrifuge at 4°C at 300 × *g* for 5 min and discard supernatant.
13. Resuspend pellet in 500 μL (per well) of pre-warmed TrypLE.
14. Place in water bath at 37°C for 5 min.
15. Flick after 5 min and when you can still see RGF BME floating in solution, incubate at 37°C for additional 5 min.
16. Forcefully pipette up and down to dissolve any last RGF BME and resuspend cells.
17. Transfer 50 μL of this solution into a 96-well plate to assess single cell dissociation under the microscope. If you have clumps of 1–10 cells you can proceed. If clumps are still bigger, prolong incubation for another 5 min, flick, and check again.
18. Add pre-warmed Advanced DMEM/F-12 supplemented with 10% Fetal Bovine Serum and Penicillin/Streptomycin (1:100 dilution) to the 15 mL tube containing the dissociated organoids in TrypLE. Use at least the same volume of Advanced DMEM/F-12 supplemented with 10% Fetal Bovine Serum and Penicillin/Streptomycin (1:100 dilution) as TrypLE was used (500 μL per well of organoids).
19. Centrifuge at 4°C at 300 × *g* for 5 min, discard supernatant, and resuspend the pellet in 400 μL organoid medium **without antibiotics**.
20. Transfer 200 μL of cell suspension into each well of a 48-well plate, let the cells sink to the bottom and analyze cell density under the microscope. The confluency should be around 80%.
21. Keep plate with dissociated organoids in 37°C incubator until Lipofectamine-RNP complexes are ready (~30 min; step 24). Proceed to step 22 immediately.

Transfection of organoids with RNP complexes

Timing: 1 day

This section details the transfection of the murine GC organoids with the previously assembled RNP complexes using Lipofectamine RNAiMAX transfection reagent.

22. For each well of the 48-well plate used for transfection, combine the following:

Reagent	Amount per well
RNP (steps 6 and 7)	50 µL
Lipofectamine RNAiMAX transfection reagent	2.4 µL
Opti-MEM	47.6 µL
Total	100 µL

23. Incubate at 20°C–25°C for 20 min.
24. Add the 100 µL Lipofectamine-RNP complexes gently to one well of dissociated organoids.
25. Incubate the plate for 24 h in a 37°C tissue culture incubator.

Troubleshooting 2: If organoid cells die within 24 h of incubation with transfection mix, keep organoids in the transfection mix for a shorter time.

Troubleshooting 3: If organoid cells die from exposure to lipofectamine, nucleofection can be attempted instead.

Sorting cells containing RNP complexes

Timing: 3 h

In this section, we report how to sort organoid cells that are positive for the ATTO™ 550 fluorescent marker incorporated in the tracrRNA and therefore, presents a surrogate measure successful RNP complex transfection.

Note: We recommend sorting the live, ATTO™ 550-positive cells in two different ways. First, sort these cells in two 96-well tissue culture plates with one cell per well. This will give you a very high confidence that any outgrowing organoids were derived from one cell clone. Secondly, single sort the cells into an Eppendorf tube. This step provides reduced confidence that organoids were derived from one cell clone but has a significantly higher chance of organoid outgrowth.

26. Collect all the transfected cells in a 15 mL tube.
27. Centrifuge at 4°C at 300 × *g* for 5 min and discard supernatant.
28. Resuspend the pellet in 500 µL of FACS buffer without antibiotics. Prepare FACS buffer fresh on the day of use and store at 4°C until use.
29. Pass cell suspension through a 40 µm filter and transfer into a FACS tube.
30. Add 0.5 µL of SYTOX™ Blue viability dye to 500 µL of single-cell suspension in FACS buffer (1:1000 dilution) right before fluorescence-activated cell sorting and mix by pipetting.
31. Via fluorescence-activated cell sorting, sort single live cells highly positive for tracrRNA-ATTO™ 550 (540–565 nm excitation; ~600 nm emission) into wells (one cell per well) of a 96-well plate containing 30 µL of organoid medium supplemented with Penicillin/Streptomycin (1:100 dilution) (see gating strategy in Figure 3).

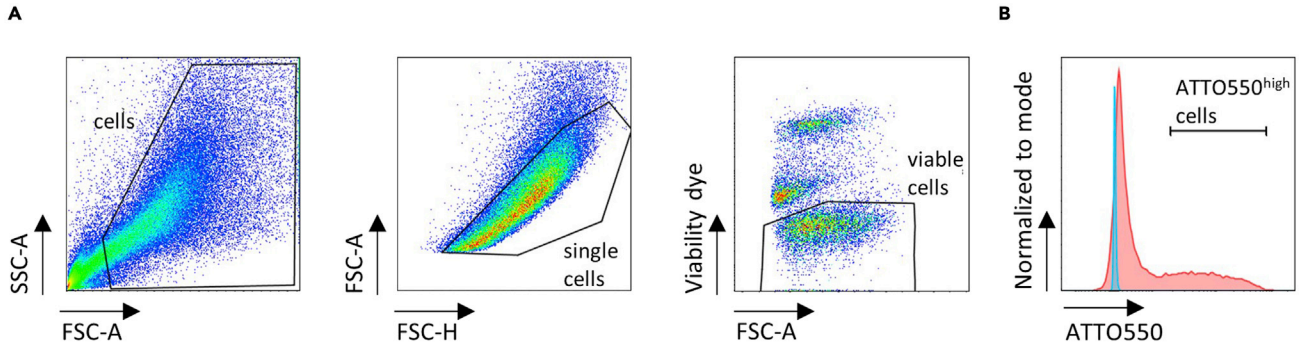

Figure 3. Gating strategy for single cell sorting of ATTO 500+ cells

(A) Gating strategy used to sort viable ATTO 550^{high} cells from organoid cells transfected with RNP complexes.

(B) Overlay histogram of cells transfected with RNP complexes (red) and mock transfected cells (RNPs without gRNA) (light blue) to demonstrate high ATTO 550 positivity.

32. Add 60 μL of RGF BME to each well and resuspend.
33. Place plate into incubator to let RGF BME become solid.
34. Add 100 μL of organoid medium supplemented with Penicillin/Streptomycin (1:100 dilution) to each well.
35. Place the plate in a 37°C incubator.

Additionally:

36. Using fluorescence-activated cell sorting, collect between 10,000 and 20,000 live ATTO™ 550 highly positive cells into one Eppendorf tube containing 500 μL of organoid medium supplemented with Penicillin/Streptomycin (1:100 dilution) (see gating strategy in Figure 3).
37. Centrifuge at 4°C at 300 × *g* for 5 min and discard supernatant.
38. Resuspend organoids in RGF BME at 50 cells / μL RGF BME ratio and pipette 50 μL of the cell-RGF BME suspension into five wells of a pre-warmed 24-well plate.
39. Place the plate in a 37°C incubator for 10 min to solidify the RGF BME.
40. Add 500 μL of organoid medium supplemented with Penicillin/Streptomycin (1:100 dilution) to each well.
41. Place the plate in a 37°C incubator.

Note: Generally, sorted cells derived from dissociated murine GC organoids re-generate organoids after a few days and the organoids recover their typical morphology and size 7–10 days after sorting.

Note: The transfection efficiency using the Lipofectamine RNAiMAX Transfection Reagent (ThermoFisher) was quite consistent between our experiments.

Manual organoid "picking" to establish clonal cultures

Timing: 2–3 weeks

This section portrays the manual "picking" of organoids from bulk single-sorted cells (steps 35–40). This is an alternative to the single cell in 96-well sorting method to establish clonal cultures. We recommend the use of this process in addition to the "single cells in 96-well" approach. This ensures establishment of a high number of organoids harboring mutations. It increases chances of multiple knockout clones, even in cases were outgrowth of clones from single cells fails or where the frequency of knockout clones is low ("hard-to-CRISPR" target genes). We note here, that "hand-picked"

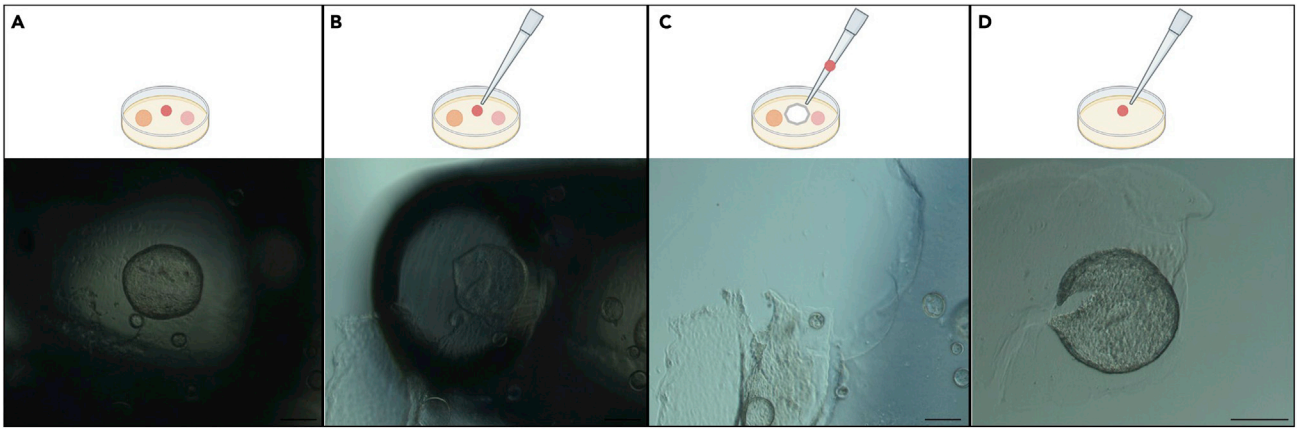

Figure 4. Manual "picking" of organoids

(A) Shows an organoid grown in RGF BME dome surrounded by other organoids.

(B) The image shows a 1,000 μL pipette tip placed over (dark ring surrounding the organoid) an organoid immediately prior "picking" of that organoid by pipetting it into the pipette tip.

(C) A micrograph showing the now empty place in the RGF BME dome where the picked organoid was.

(D) Presents an image of the manual picked organoid in the new well confirming that only one organoid was picked. Scalebars represent 200 μm.

organoids, which initially outgrew from domes seeded with 2,500 sorted cells / dome (step 38) have an increased risk of not being clonal but could be derived from 2 or more cells of origin.

42. Seven to ten days after sorting, manually pick single organoids under the microdissection microscope (Figure 4 and Methods Video S1).
    a. Prepare multiple 1.5 mL Eppendorf tubes, each containing 100 μL organoid medium supplemented with Penicillin/Streptomycin (1:100 dilution).
    b. Keep the tubes on ice.
    c. Under the microdissection microscope and using a 1,000 μL pipette with a filter tip, pick single organoids out of the RGF BME domes.
    d. Individually transfer picked single organoids into new wells for visual inspection before transferring to the prepared Eppendorf tubes with the organoid medium and keep on ice while picking more single organoids.
    e. Pick between 15 and 30 single organoids.

Note: We found that manual picking of 15–30 single organoids is optimal as generally, 5 clonal organoid cultures out of 15 manually picked organoids harbored the desired knockout.

43. Centrifuge at 4°C at 300 × *g* for 5 min and carefully discard supernatant by pipetting. Leave a little bit of media and visually confirm that the single organoid remains in the tube.
44. Resuspend each organoid in 50 μL RGF BME and pipette into one well of a pre-warmed 24-well plate.
45. Place the plate in a 37°C incubator for 10 min to solidify the RGF BME.
46. Add 500 μL of organoid medium supplemented with Penicillin/Streptomycin (1:100 dilution).
47. Place the plate in a 37°C incubator.
48. Passage each clonal organoid culture normally.

Loss of gene of interest confirmation via Western blotting

Timing: 3 days

To confirm the knockout of our GOI at the protein level, we conducted a Western blot as soon as we were able to grow sufficient organoids (approximately 3,000) to extract protein (Figure 5).

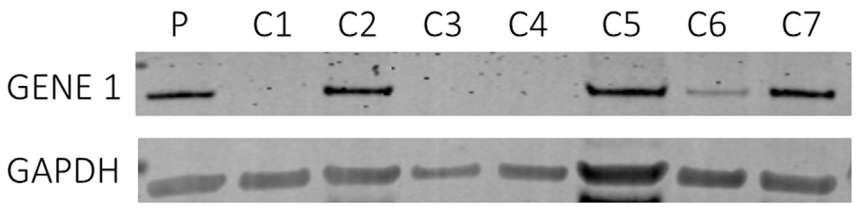

Figure 5. CRISPR knockout confirmation via Western blot analysis

Western blot analysis for both a CRISPR target protein and control GAPDH. P = parental organoids; C = clonal organoids. C1, C3 and C4 represent successful complete knockouts, whereas C6 represents an incomplete knockout meaning strongly reduced protein level suggesting heterozygous loss of target gene.

For more background on the technique, theory, and troubleshooting, please look at this comprehensive review by Mahmood and Yang.[22]

49. Lyse murine organoid cells to extract protein. In this protocol, we are using the widely available TrypLE solution to dissociate organoids and break down RGF BME before collecting pellets for protein lysates. However, other methods of dissociation and collecting organoid pellets for protein lysates can be used instead.

Note: When using TrypLE solution to break down the embedding protein matrix, residual protein contamination from the matrix might affect the Western Blot quality.

    a. Prepare single-cell suspension from approximately 3,000 organoids using TrypLE (steps 8–16 of section "Preparation of organoids for lipofection").
    b. Centrifuge single-cell suspension at 4°C at 1500 300 × *g* for 5 min and discard supernatant.
    c. Resuspend pellet in 50 µL RIPA lysis buffer containing protease and phosphatase inhibitors and transfer to a 1.5 mL Eppendorf tube. RIPA lysis buffer can be prepared in advance, aliquoted and stored at −20°C for up to 6 months. Add protease (1 tablet per 10 mL lysis buffer) and phosphatase (1 tablet per 10 mL lysis buffer) inhibitors directly before use.
    d. Keep on ice for 30–60 min and vortex 3 times in between.
    e. Centrifuge at 4°C at 13,000 × *g* for 20 min.
    f. Carefully transfer supernatant to new, pre-cooled Eppendorf tube.

Pause point: You can store the protein lysates long-term at −80°C.

50. Determine protein concentration via Pierce™ BCA Protein Assay following the manufacturer's instructions (https://www.thermofisher.com/document-connect/document-connect.html?url=https%3A%2F%2Fassets.thermofisher.com%2FTFS-Assets%2FLSG%2Fmanuals%2FMAN0011430_Pierce_BCA_Protein_Asy_UG.pdf).
51. Run the electrophoresis.
    a. Use 35 µg protein per lane and add 1× NuPAGE LDS sample Buffer and 1× NuPAGE Sample Reducing Agent. Equalize the volume in each lane to 40 µL with distilled H_2O.
    b. Heat samples at 90°C for 10 min.
    c. Pour 1× MES running buffer into electrophoresis tank.
    d. Place Novex NuPAGE Tris-Acetate Mini gel inside the electrophoresis tank, ensure the buffer covers the gel completely and carefully remove comb.
    e. Load Precision Plus Protein Standards Kaleidoscope marker (10 µL) and samples (40 µL) into each well.
    f. Run gel at 20°C–25°C at 120 V for approximately an hour.
52. Run the electrotransfer.

a. Separately wet sponges and 6 filter papers in transfer buffer and the PDVF membrane in methanol. Whereas 10× transfer buffer can be prepared in advanced and stored at 20°C–25°C for up to 6 months, 1× transfer buffer should be prepared on the day of use at least 30 min before use. Add 20% methanol to 1× transfer buffer on the day of use. Store at 4°C until use.
b. Retrieve the gel from the plastic plates.
c. Create a transfer sandwich in which the sponge is followed by three filter papers, the gel, the PVDF membrane, three filter papers and another sponge. Ensure there are no air bubbles between the layers.
d. Put the sandwich into the transfer apparatus, add transfer buffer and place it on ice. Close the apparatus with the lid connected to the electrodes. The PVDF membrane needs to be placed between the gel and the positive electrode.
e. Transfer at 4°C for 16–18 h (42 V, 90 mA).

53. Block the membrane with 5% skim milk powder in TBST at 20°C–25°C for 1 h. Prepare 10× TBS in advance and store at 20°C–25°C for up to 6 months. Prepare 1× TBS and add 0.1% Tween 20 detergent to make TBST buffer. TBST buffer can be stored at 20°C–25°C for up to 6 months.
54. Incubate membrane with primary antibody against your protein of interest in 1% skim milk powder in TBST at 4°C for 16–18 h.
55. Wash membrane 3 times with TBST at 20°C–25°C for 5 min.
56. Incubate membrane with primary antibody against housekeeping protein in 1% skim milk powder in TBST at 20°C–25°C for 1 h.

Note: As a reference housekeeping protein, we used GAPDH (anti-GAPDH antibody, Mouse monoclonal, clone GAPDH-71.1, 1:2000 dilution).

57. Wash membrane 3 times with TBST at 20°C–25°C for 5 min.
58. Incubate membrane with fluorescence labeled secondary antibodies in 50% Intercept buffer in TBST at 20°C–25°C for 1 h.

Note: We used either IRDye 680RD Goat anti-Mouse IgG (H+L) or IRDye 800CW Goat anti-Rabbit IgG (H+L) depending on the species of the primary antibodies. Both secondary antibodies were used at a 1:10,000 dilution.

59. Scan the membrane with an Odyssey Imager from LI-COR to detect the signal.

Note: If knockout organoids are being cultured for an extended period of time or being utilized in long- term experiments, we suggest to double check target protein knockout via Western Blotting to ensure that a complete knockout status is maintained.

Optional: Sanger sequencing can be performed to validate the gene editing on the DNA level.

Troubleshooting 4: If no knockouts were generated, different crRNAs or even multiple crRNAs could be used instead.

Troubleshooting 5: If no knockouts were generated even after numerous attempts with different crRNAs or even multiple crRNAs, the GOI could be an essential gene.

EXPECTED OUTCOMES

We describe a straightforward protocol for the generation of GOI knockout organoids. The gene-editing strategy we utilize here is based on transient expression of the editing complex, eliminating the need of establishing stably expressing Cas9 organoid cultures before being able to knockout the

Table 1. Percentage of viable cells and ATTO 550+ cells after transfection with RNP complexes

Target	% Viable cells / total cells	% ATTO 550+ cells / live cells
Mock	44.1	N/A
GOI 1	40.3	30.7
GOI 2	32.3	26.1
GOI 3	36	49.9
GOI 4	57.6	39.2

Percent of viable cells of total cells and percent of ATTO 550+ cells of live cells after transfection with RNP complexes. Data for one mock transfection (RNP complexes contained no gRNA) and transfection of RNP complexes targeting 4 different genes is shown. FACS gating strategy for viable and ATTO 550+ cells is shown in Figure 2.

GOI.[23] Importantly, this protocol is designed to minimize the risk of off-target gene editing, introduction of transcriptional variety, and potential immunogenetic effects associated with prolonged expression of the Cas9 protein.[13–15] While we have not experimentally tested for off-target editing and target independent phenotype change frequencies, the transient presence of the editing complex and the Cas9 enzyme in the organoids in our method removes the main factors causing off-target alterations.[12–17,24] Therefore, this protocol is well suited to generate syngeneic parental / GOI knockout organoid culture pairs, for the subsequent analysis of developmental, immunological and tumor biology processes either via *in vitro* or *in vivo* experiments. Possible assays include genetic, molecular, and biochemical characterization, organoid - immune cell co-culture assays or *in vivo* transplantation into mice to address numerous biological questions. While we demonstrate the feasibility of the protocol by successful establishment of four GOI knockouts using two different gastric cancer organoid cultures, we expect the protocol to be universally applicable to any robustly growing organoid culture of interest.

The protocol includes a few critical steps at which efficiency of the process can be determined. For best results, starting cultures should be in the exponential growth phase (Figure 1). After steps 1–25 of the protocol, the organoids are to be single-cell sorted by fluorescent-activated cell sorting (FACS) for ATTO 550 positivity, as a surrogate marker for successful transfection of the editing complex. The FACS step allows quantification of the viability of the organoids post transfection (see gating strategy in Figure 2) and the efficacy of transfection (Table 1). In our hands, 32%–58% of dissociated organoid cells were viable, of which 30%–50% were highly positive for the ATTO 550 marker (Table 1).

The outgrowth efficacy of organoids after cell sorting, represented by the percentage of organoids growing from the total number of wells of 96-well plates or the percentage of organoids growing from the total number of sorted cells, may widely differ. We introduce two supplementary methods to ensure that organoids are outgrowing. Either single cells are sorted in individual wells of 96-well plates (step 30) or single cells are pool/bulk sorted into a vial and plated into domes at a cell density of 2,500 cells per dome (steps 35–40). Method 1 ensures that outgrowing organoids are single cell derived clones but generate only a low number of organoid cultures with an outgrowth rate between 1%–3% (Table 2). In Method 2, many more cells can be seeded, and therefore a comparable outgrowth rate of 1%–8% leads to high numbers of organoids (Table 2).

Subsequently, these can be manually picked (step 42, Figure 4, Methods Video S1), to establish organoid cultures of clonal origin. As the final check of successful gene-editing of the GOI, we perform protein immunoblotting to confirm loss of target protein, an example is provided in Figure 5. For our example gene edits, we had 6%–40% of tested organoid cultures of clonal origin having complete loss of the target protein and 9%–40% a significantly reduced protein level compared to parental organoids (Table 3). At this high editing rate, it is very feasible to establish and utilize not only parental / complete knockout pairs of clonal organoid cultures for subsequent experimentation but also parental / complete / heterozygous loss mutant trios, if desired.

Table 2. Percentage of organoid outgrowth from single-sorted ATTO 550+ cells

Target	% Outgrowth from 96 well plate (#organoids /# total wells)	% Outgrowth from bulk sorted cells (# organoids / # total sorted cells)
GOI 1	1.04 (3/288)	NA
GOI 2	2.08 (2/96)	4.33 (525/12130)
GOI 3	2.08 (2/96)	0.99 (160/16221)
GOI 4	3.13 (3/96)	7.89 (250/3170)

Percent of organoid outgrowth from single-sorted ATTO 550+ cells. The first column shows the outgrowth percentage of organoids as defined by the number of organoids growing from single-sorted cells in single wells of 96-well plates. The second column shows the outgrowth percentage as the number of organoids growing from bulk single-sorted cells. Outgrowth data is shown for four different experiments targeting four different genes.

Taken together, we describe here a simple to follow, versatile and broadly applicable CRISPR-Cas9-mediated gene editing protocol for organoids, which is strongly mitigating risks of off-target genetic and phenotypic alterations. These attributes make our protocol an excellent choice to study GOI knockout effects in the context of many biological processes and phenomena.

LIMITATIONS

This protocol is not suitable for CRISPR-Cas9-mediated high through-put screening efforts as the editing efficacy is not sufficient and the organoid handling times are too high. For screening efforts typically viral delivered, permanent Cas9-expressing cell lines or organoid cultures are being used.[23,25] Our protocol utilizes transient expression and activity of gene editing complexes. While our protocol has a lower editing efficacy compared to permanent Cas9 expression methods, the transient presence of the editing components is known to reduce off target editing and mitigates the risk of Cas9-caused phenotypical changes, such as increased immunogenicity.[12–17,24]

This protocol is suited to generate gene knockout organoids. If base -editing or gene knock-in manipulations are desired this protocol may be adapted or alternative protocols, employing similar methodology, maybe used as described in the literature.[2,7]

This protocol and many other CRISPR-Cas9-editing protocols require robust growth potential of the organoids over several passages. Organoids which grow at a very slow rate, or which stop to proliferate after approximately five or less passages will not be suitable. This is because the outgrowth of clones after cell sorting and clonal expansion for GOI knockout confirmation by western blot requires continuous and robust organoid proliferation.

The outgrowth efficacy of organoids from single cells sorted in individual wells of 96-well plates may differ strongly for different organoid cultures. The single cell environment does pose a stress on the cells and without the support from niche cells outgrowth capacity will be different for every organoid culture. To ensure that sufficient organoids are outgrowing we introduce a second supplementary method. Next to single cells sorted in individual wells of 96-well plates, single cells are pool/bulk sorted into a vial and plated into domes at a cell density of 2,500 cells per dome. With this method, many more cells can be seeded, and therefore a comparable outgrowth rate of 1%–8% leads to high numbers of organoids, which subsequently can be handpicked for organoid culture expansion.

TROUBLESHOOTING

Problem 1

Organoids grow slower than usual or expected.

Potential solution

The primary cause for unusual slow growth of organoids is suboptimal or old growth medium. Prepare fresh medium and ensure that all required components of your organoid growth medium are added. In addition, the medium can be exchanged more frequently (every 2 days). We describe the use of IntestiCult™ Organoid Growth Medium because we observe consistent growth of our

Table 3. Percentage of organoid cultures with complete or incomplete knockout

Target	% Complete knockout rate (# complete knockouts /# tested clones)	% Incomplete knockout rate (# incomplete knockouts /# tested clones)
GOI 1	6.25 (2/32)	9.38 (3/32)
GOI 2	33.33 (5/15)	26.67 (4/15)
GOI 3	6.67 (1/15)	26.67 (4/15)
GOI 4	40.00 (2/5)	40.00 (2/5)

Percent of complete and incomplete knockout of tested organoid clonal cultures is presented as defined by the number of complete (column 1) or incomplete (column 2) knockouts per all tested organoid clones. Incomplete knockouts are defined as where protein levels are reduced >= 50% of control parental levels but is not completely lost. Incomplete protein loss is an indicator of heterozygous gene editing. See Figure 5 for examples of complete and incomplete knockouts.

murine GC organoids. However, any other media in which your organoids of choice are known to grow robustly, might be used alternatively.

Problem 2

Organoid cells die within 24 h of incubation with transfection mix (step 25).

Potential solution

Shorten the incubation time. We incubated dissociated organoids with the Lipofectamine-RNP complexes for as little as 4 h and successfully generated a knockout. However, transfection efficiency is reduced with a shorter incubation time.

Problem 3

Organoid cells die from exposure to lipofectamine reagent (step 25).

Potential solution

If organoids die from exposure to lipofectamine reagent, the use of lipofectamine in lower concentrations can be attempted first.

Alternatively, organoid cell death induced by the lipofectamine reagent can be avoided by using nucleofection rather than transfection with lipofectamine. Ongoing optimization of the protocol in our lab suggests that a similar or even higher transfection efficiency can be achieved by using nucleofection.

Problem 4

No knockouts were generated (step 58).

Potential solution

There are several reasons for not generating a knockout. The gRNA itself could have a low genome editing activity e.g., it could fail to induce a double-strand break. Our recommendation is to design more than just one crRNA to create more than one gRNA. Also, multiple gRNAs could be used in a single transfection. Another possibility could be that un-edited organoid clones were picked. To mitigate that risk, the number of manually picked organoids can be increased.

Problem 5

No knockouts were generated even after employing different or multiple gRNAs (step 58 and problem 4).

Potential solution

If no knockouts were generated after numerous attempts using different or multiple gRNAs, the GOI could be an essential gene, where the gene knockout leads to death or growth impairment of the organoids. To test if the GOI is an essential gene, different methods could be employed, such as the use of RNAi to create a knockdown[26] or generating a conditional knockout for the GOI.[27,28]

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Moritz F. Eissmann (Moritz.eissmann@onjcri.org.au).

Materials availability

Reagents generated within this study are available upon request.

Data and code availability

This study did not generate any unique datasets or codes.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2023.102076.

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AUTHOR CONTRIBUTIONS

A.H., M.E., and M.F.E. conceived and designed experiments; A.H., C.D., and M.F.E. performed experiments and analyzed the data. A.H. wrote the manuscript; A.H., C.D., M.E., and M.F.E. reviewed and edited the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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