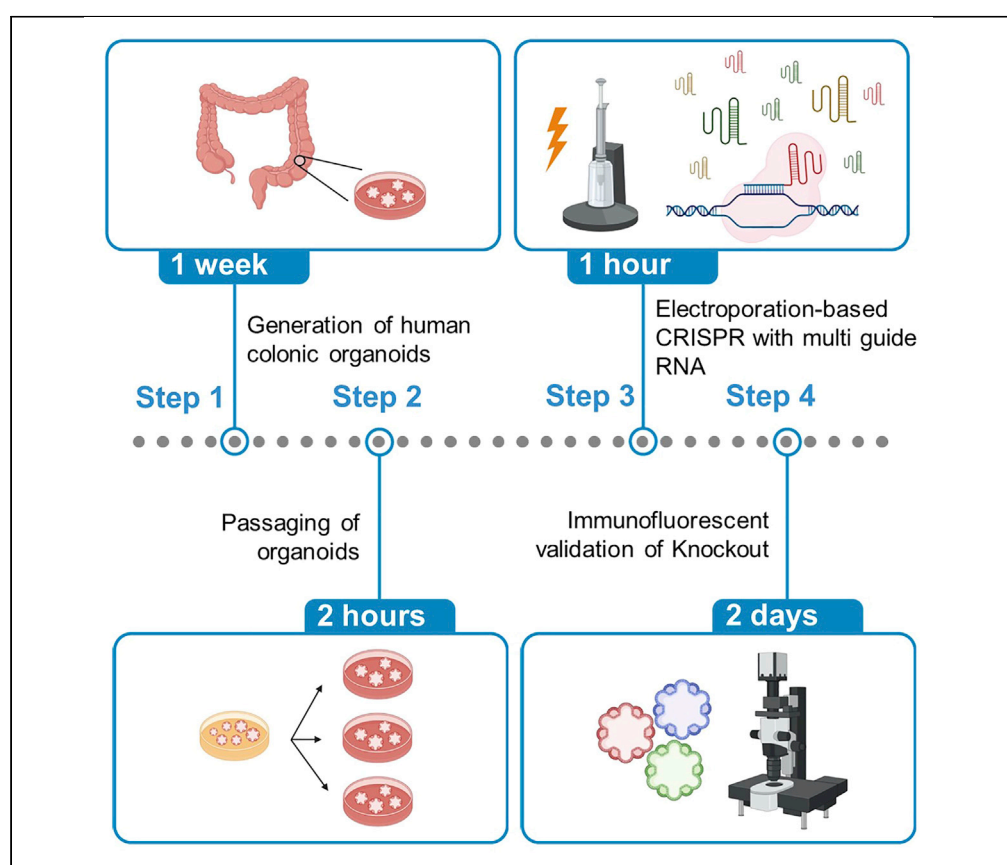


Protocol

Generation and immunofluorescent validation of gene knockouts in adult human colonic organoids using multi-guide RNA CRISPR-Cas9



While readily achieved in cell lines, the application of CRISPR-Cas9 gene editing in human-derived organoids suffers from limited efficacy and complex protocols. Here, we describe a multi-guide RNA CRISPR-Cas9 gene-editing protocol which efficiently achieves complete gene knockout in adult human colonic organoids. This protocol also describes crucial steps including how to harvest patient tissue to maximize gene-editing efficacy and a technique to validate gene knockout following editing with immunofluorescent staining of the organoids against the target protein.

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

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Highlights

Derivation of adult stem-cell-derived human colonic organoids

Generation of target gene knockout using multi-guide RNA CRISPR-Cas9

Immunofluorescent validation of target gene knockout for organoids

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Protocol

Generation and immunofluorescent validation of gene knockouts in adult human colonic organoids using multi-guide RNA CRISPR-Cas9

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SUMMARY

While readily achieved in cell lines, the application of CRISPR-Cas9 gene editing in human-derived organoids suffers from limited efficacy and complex protocols. Here, we describe a multi-guide RNA CRISPR-Cas9 gene-editing protocol which efficiently achieves complete gene knockout in adult human colonic organoids. This protocol also describes crucial steps including how to harvest patient tissue to maximize gene-editing efficacy and a technique to validate gene knockout following editing with immunofluorescent staining of the organoids against the target protein.

BEFORE YOU BEGIN

Organoid culture models recapitulate *ex vivo* niche factors and the basement membrane necessary to maintain and propagate tissue-resident stem cells.¹ When combined with CRISPR-Cas9 genome editing technology, organoids are a powerful tool which can model various disease-states. CRISPR-Cas9 genome engineering via lentiviral transduction is an effective method but requires multiple time-consuming steps including cloning a single guide RNA (sgRNA) into a plasmid vector, generation of lentivirus particles and transduction of the target cell, commonly taking 2–3 months in total.²

The ribonucleoprotein (RNP) electroporation approach is a more time-efficient method of CRISPR-Cas9 genome editing but suffers from low knockout (KO) efficacy, as the RNP complex is transient and degraded within 24–48 h. While genome engineering of canonical driver mutations in colorectal cancer such as *APC*, *KRAS*, *SMAD4* and *TP53* may be performed using a plasmid electroporation approach,³ this is greatly aided by the presence of selection media which only permits successfully edited cells to survive. RNP electroporation for mutations which lack such defined selection media suffers from low transfection efficacy.

The protocol below describes the introduction of genetically engineered mutations which lack a selection media using a multi-guide CRISPR-Cas9 RNP electroporation approach. *FBXW7* has been chosen as the target gene, but this protocol may be applied to any other gene for which there is no selection media available.

Institutional permissions

Informed consent was obtained from all patients from whom tissue was obtained. All experiments in this study were approved by the University of Oxford – Translation Gastroenterology Unit



(16/YH/0247). Experiments involving patient-derived tissue must obtain approval by the relevant ethics committee in the host institution prior to commencement.

Preparation of harvesting solution and human conditioned media

⌚ Timing: 2 h

1. Prepare FBS/PBS solution.
2. Prepare EDTA harvesting solution, filter using a 0.22 μ m vacuum filter, and store at 4°C until required.
3. Prepare Organoid passaging solution and store at 4°C until required.
4. Prepare Conditioned media buffer and store at 4°C until required.
5. Prepare Human conditioned media, aliquot to 25 mL, and store at –20°C until required. This has a shelf-life of about 2 months.

⚠ **CRITICAL:** Repeated freeze-thaw cycles of media components severely affect the efficacy of the reagent and should be minimized by aliquoting.

⚠ **CRITICAL:** 10 M NaOH must be handled within a chemistry fume hood and with full protective equipment. Avoid inhalation of fumes and contact with skin and clothing.

Alternatives: Commercially ready IntestiCult™ which comprises all the necessary components for organoid growth can be used.

Ordering of multi-guide RNA

⌚ Timing: 15 min

6. Select gene of interest from <https://www.synthego.com/products/crispr-kits/gene-knockout-kit>.

Alternatives: The multi-guide RNA when ordered commercially will be shipped as one tube containing all three individual sgRNAs. It is possible to purchase the sgRNAs individually and to reconstitute them subsequently.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
FBXW7/Cdc4 Antibody (1:100)	Novus Biologicals	Cat# H00055294-M02
Goat Anti-Mouse IgG H&L (DyLight® 550) (1:500)	Abcam	Cat# ab96872
Biological samples		
Normal adult human colonic tissue	Churchill Hospital, Oxford, UK.	N/A
Chemicals, peptides, and recombinant proteins		
DPBS, no calcium, no magnesium	Gibco	Cat# 14190144
Fetal bovine serum, certified, heat inactivated	Gibco	Cat# 10082147
HBSS, no calcium, no magnesium	Gibco	Cat# 14170112
EDTA	Invitrogen	Cat# 15576028
NaOH, 10 M	Sigma-Aldrich	Cat# 72068
Advanced DMEM/F12	Gibco	Cat# 12634010
GlutaMAX Supplement	Gibco	Cat# 35050061

(Continued on next page)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
HEPES, 1 M buffer solution	Gibco	Cat# 15630049
Penicillin-streptomycin (10,000 U/mL)	Gibco	Cat# 15140122
B-27 Plus Supplement (50 ×)	Gibco	Cat# A3582801
SB 202190	Tocris	Cat# 1264
SB 431542	Tocris	Cat# 1614
Prostaglandin E ₂	Tocris	Cat# 2296
Recombinant human noggin	Peprotech	Cat# 120-10C
Animal-free recombinant human EGF	Peprotech	Cat# AF-100-15
Nicotinamide	Sigma-Aldrich	Cat# 240206
N-acetylcysteine amide	Sigma-Aldrich	Cat# A0737
Y27632	Tocris	Cat# 1254
Bovine serum albumin	Sigma-Aldrich	Cat# A7906
Triton X-100	Sigma-Aldrich	Cat# X100
Saponin	Sigma-Aldrich	Cat# 47036
Goat serum	Gibco	Cat# 1620064
Paraformaldehyde fixative solution	Thermo Scientific	Cat# J61984.AP
Experimental models: Cell lines		
Mouse: L Wnt-3A	ATCC	CRL-2647
Mouse: HA-R-Spondin1-Fc 293T	Cultrex	Cat# 3710-001-01
Oligonucleotides		
FBXW7 multi-gRNA sequences: GCAAGGAATGGTGAAGTTGT GATGAATCGTGTGGTAGAGG AGCAAAAGACGACGAAGTGG	Synthego	
Software and algorithms		
ICE analysis	Synthego	https://ice.synthego.com/#/
Other		
0.22 μm pore bottle top vacuum filter	Corning	Cat# 431118
15 mL conical centrifugal tubes	Falcon	Cat# 352097
50 mL conical centrifugal tubes	Falcon	Cat# 352098
1.5 mL microcentrifuge tubes	VWR	Cat# 525-0990
24-well cell culture plate	Sarstedt	Cat# 83.3922
20 μL pipette tips	Starlab	Cat# S1123-1810
200 μL pipette tips	Starlab	Cat# S1120-8810
1,000 μL pipette tips	Starlab	Cat# S1122-1830
P20 pipette	Gilson	Cat# F123600
P200 pipette	Gilson	Cat# F123601
P1000 pipette	Gilson	Cat# F123602
100 mm cell culture dish	Corning	Cat# 430167
Cultrex Reduced Growth Factor Basement Membrane Extract, Type 2, Pathclear	Bio-Techne	Cat# 3533-005-02
Cultrex Organoid Harvesting Solution	Bio-Techne	Cat# 3700-100-01
Transfer pipettes	VWR	Cat# 612-4491
Dragonfly High Speed Confocal Microscope System	Oxford Instruments	
Neon Transfection System	Invitrogen	Cat# MPK5000

MATERIALS AND EQUIPMENT

FBS/PBS solution		
Reagent	Final concentration	Amount
DPBS (no Ca ²⁺ /Mg ²⁺)	–	450 mL
FBS	–	50 mL
Total	N/A	500 mL

Note: Store at 4°C for 3 months.

EDTA harvesting solution		
Reagent	Final concentration	Amount
HBSS (no Ca ²⁺ /Mg ²⁺)	–	499.5 mL
EDTA	13 mM	1.85 g
NaOH, 10 M	10 mM	500 µL
Total	N/A	500 mL

Note: Store at 4°C for 3 months.

Organoid passing solution		
Reagent	Final concentration	Amount
Advanced DMEM/F-12	–	490 mL
GlutaMAX supplement	1 ×	5 mL
HEPES buffer solution	10 mM	5 mL
Total	N/A	500 mL

Note: Store at 4°C for 3 months.

Conditioned media buffer		
Reagent	Final concentration	Amount
Advanced DMEM/F-12	–	485 mL
GlutaMAX supplement	1 ×	5 mL
HEPES buffer solution	10 mM	5 mL
Penicillin-Streptomycin	100 U/mL	5 mL
Total	N/A	500 mL

Note: Store at 4°C for 3 months.

Human Conditioned Media		
Reagent	Final concentration	Amount
L-Wnt-3A conditioned medium	50% (v/v)	25 mL
R-spondin conditioned medium	20% (v/v)	10 mL
B-27 plus supplement	1 ×	1 mL
SB 202190	10 µM	50 µL
SB 431542	0.5 µM	5 µL
Prostaglandin E ₂	1 µM	5 µL
Noggin	50 ng/mL	2.5 µL
EGF	50 ng/mL	2.5 µL
Nicotinamide	10 mM	61 mg
N-acetylcysteine amide	1.25 mM	10 mg
Conditioned media buffer	–	13.935 mL
Total	N/A	50 mL

Note: Store at 4°C for 3 months.

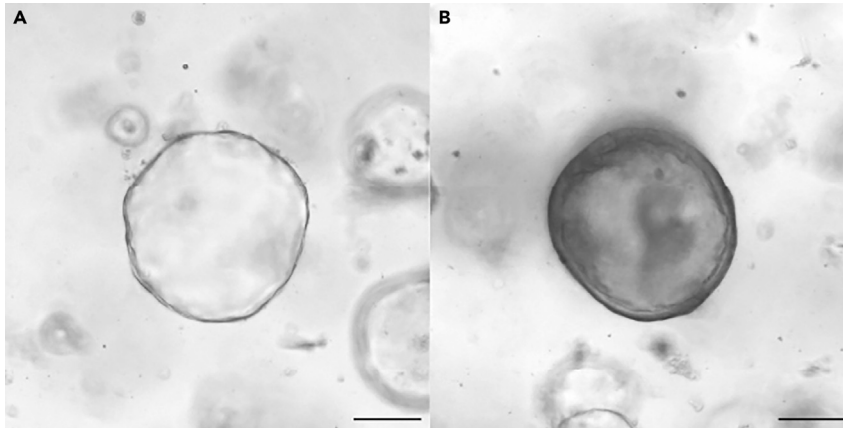


Figure 1. A comparison of cystic and differentiated organoids

(A and B) Cystic organoids have the best growth potential and are required to perform gene editing with high efficacy. The organoids in (A) represents a cystic organoid with a thin wall and minimal extrusion into the core of the organoid. In contrast, the organoid in (B) has a thick wall and is darker, representing a differentiated organoid. Scale bar equals 100 μm .

Alternatives: Commercially prepared IntestiCult Organoid Growth Medium (Human) (StemCell Technologies, Cat# 06010) can also be used.

STEP-BY-STEP METHOD DETAILS

Generation of human colonic organoids

⌚ Timing: 1 week

Adult colonic organoids become less cystic at advanced passages and their ability to be expanded between passages decreases. This usually occurs from passage number 8 and beyond (Figure 1). Late-passage organoids also lose gene editing efficacy. We recommend using fresh biopsy specimens for each gene editing attempt to maximize efficacy, and to allow for onward experimental procedures.

1. Warm EDTA harvesting solution to 37°C.
2. Ensure a water bath has equilibrated to 37°C.
3. Specimen collection.
 - a. A 1 × 1 cm surgical specimen of normal colonic tissue is sufficient for harvesting tens of thousands of crypts.
 - b. Minimize warm ischemia time by placing the surgical tissue in cold FBS/PBS solution, and transporting the specimen to the laboratory in an ice box.

Note: The addition of FBS provides serum support to the tissue before plating and improves culture efficiencies compared to PBS alone.

4. Wash the specimen in 25 mL of FBS/PBS in a 50 mL centrifuge tube and gently agitate the solution. Repeat twice.
5. Transfer the specimen into 25 mL of EDTA harvesting solution in a 50 mL centrifuge tube and submerge the entire centrifuge tube in the water bath.
 - a. Beginning at 0 min, shake the tube on a vortex or by hand.
 - b. Repeat every 2 min for a total of 10 min. Crypts from the colonic tissue will be sloughed off into the EDTA harvesting solution.

- c. Transfer the specimen into a new 50 mL centrifuge tube containing 25 mL of EDTA harvesting solution.
- d. Add 25 mL of FBS/PBS to the previous EDTA harvesting solution containing crypts and store in an ice box.
- e. Repeat shaking of the new EDTA harvesting solution as per steps 5a and 5b.
- f. Remove the specimen and discard.
- g. Add 25 mL of FBS/PBS to the second EDTA harvesting solution with crypts.
6. Centrifuge both EDTA harvesting solution at 400 g × 6 min at 4°C.
7. Remove supernatant and resuspend the pellet in 5 mL of FBS/PBS.
8. Pipette two 10 µL aliquots of the suspension to a cell culture dish and observe under the microscope.
 - a. Count the number of crypts in each aliquot and take average. This gives the concentration of crypts in the crypt suspension.
 - b. Multiple this by 500 to obtain the total number of crypts in the crypt pellet.
 - c. Aim to plate 300–350 crypts per 40 µL of BME.
 - d. Having decided the number of crypts required in total, transfer the total volume of crypt suspension to a 15 mL centrifuge tube and top up to 5 mL with FBS/PBS.

△ CRITICAL: The majority of crypts will not survive the first few days. Plating too few crypts reduces the number of viable organoids generated. Plating too many crypts leads to competition between organoids for nutrients in the media and will require more frequent changing of media leading to increased utilization of resources and cost.

Note: 40 µL of BME is used in step 8c as a reference as this is the volume of BME used for each well in a 24-well plate. Scale up or down as required.

Alternatives: Many commercially available basement membrane extracts can be used, including Matrigel Matrix (Corning, Cat# 356255).

9. Centrifuge at 400 g × 6 min at 4°C.
10. Remove supernatant and add BME based on the afore-calculated crypt to BME ratio.
 - a. Resuspend the crypt in BME.

△ CRITICAL: Avoid the formation of air bubbles as this leads to the integrity of BME being compromised after it solidifies.

11. Place in a 37°C incubator for 30 min to allow the BME to solidify.
12. Add 250 µL of penicillin/streptomycin and 25 µL of Y27632 to each 25 mL aliquot of human conditioned media.
13. If using a 24-well plate, add 500 µL of human conditioned media supplemented with antibiotics and Y27632 to each well and return plate to the incubator.
14. Change media every 2–3 days or earlier if the media shows signs of acidification.

Note: As discussed in step 8, seeding a high density of crypts will lead to increased need for media changes. This is especially so at earlier passages as cells proliferate most rapidly. Initial signs of acidification include changing of media color from red to orange/yellow if media containing phenol red has been used, as in this protocol. Organoids will exhibit reduced growth and may not survive if grown in overly acidic environments.

15. Organoids will form by the end of 7 days and will be ready for passage.

Passaging of human colonic organoids

⌚ Timing: 2 h

This step is critical to remove crypts which failed to become organoids. Performed well, organoids following this first passage may be used for RNP electroporation.

16. Remove media from wells.
17. Place a 24-well plate in a 37°C incubator.

Note: This plate will be used at the end of the experiment for seeding of the organoids. BME solidifies at temperatures above 15°C. Warming up the plate beforehand assists the stability of the BME droplet and prevents it from dispersing across the bottom of the well by solidifying quickly. This also allows for media to be added to the well earlier.

18. If using a 24-well plate, add 500 µL of Organoid Harvesting Solution to each well and place the plate on ice for at least 45 min.

Note: Organoid harvesting solution depolymerizes the BME while leaving organoids intact. Using organoid harvesting solution ensures that the organoids can be separated more easily from the BME during centrifugation.

19. Using a P1000 pipette, aspirate the organoid harvesting solution, while breaking down any remnant BME, and transfer to a 15 mL centrifugation tube.
20. Centrifuge at 400 g × 6 min at 4°C.
21. Gently aspirate the supernatant, leaving the cell pellet undisturbed, and resuspend the solution in Advanced DMEM++.
22. Use a P200 pipette to triturate the resuspension 100 times.
23. Add advanced DMEM++ to 5 mL.
24. Centrifuge at 400 g × 6 min at 4°C again.
25. Gently aspirate the supernatant, leaving the cell pellet undisturbed, and add BME.

Note: The exact amount of BME to be added depends on the number of viable organoids. As an approximation, early passage organoids should enable expansion by 1:3 easily, whereas late passage organoids may only be expanded 1:1.5 to 1:2. Based on the amount of BME used in the previous passage, use this approximation to determine the volume of BME required.

26. Plate organoids resuspended in BME into domes in each well of the 24-well plate.
27. Return the plate to the incubator.

RNP electroporation for CRISPR-Cas9 gene editing with multi-guide RNA

⌚ Timing: 1 h

28. Set up the Neon Transfection System.

Alternatives: Alternative electroporation/nucleofection systems such as the Nucleofector™ (Lonza) or NEPA21 Super Electroporator (Nepagene) can be used.

29. Warm the following items to 37°C.
 - a. 24-well plate.
 - b. Advanced DMEM++ (no antibiotics).
 - c. Human IntestiCult Organoid Growth Media.
 - d. TrypLe Express.

Alternatives: Instead of using IntestiCult, human conditioned media as prepared previously can be used. However, this needs to be modified by removing antibiotics as the presence of antibiotics in electroporated cells can reduce its viability.

30. Set up the following:
 - a. 25 mL of Advanced DMEM++ with 25 μ L of Y27632 added.
 - b. A 15 mL centrifuge tube with 10 μ L of Y27632 added.
31. Prepare a 1.5 mL microtube with 750 μ L of the solution from step 30a.
32. Remove media from wells.

Note: In our experience, 4 wells in a 24-well plate of fully confluent organoid domes constitutes one reaction. Note the number of reactions as this impacts the volume of downstream reagents to be used.

33. Add 500 μ L of TrypLe to each well, breaking down the BME dome, and transferring the suspension to a 15 mL centrifuge tube from step 30b.
34. Submerge the centrifuge tube in a 37°C water bath for 10 min in total.
 - a. At 5 min, triturate the organoid suspension with a P1000 pipette 10 times.
 - b. Repeat at 7.5 min, and at 10 min.
35. Centrifuge at 400 $g \times 6$ min at 4°C.
36. Aspirate the supernatant and resuspend in 10 μ L/reaction of Buffer T.
37. Set up the RNP complex as follows:

RNP complex	
Reagent	Amount (μ L)
Buffer R	7.2
Cas9	1.2
Multi-guide RNA	1.6

Note: It is important to optimize the molar ratios of the Cas9 to the multi-guide RNA. In the above formula, the Cas9 and multi-guide RNA are at 25 μ M and 100 μ M concentrations respectively. The corresponding volumes give a molar ratio of 1:4, which in our experience, optimizes transfection efficacy with the amount of reagents utilized.

Note: The multi-guide RNA consists of three different gRNAs targeting a region in proximity with each other. This combination ensures a large knockout is generated. The sequences used in this protocol have been included in the [key resources table](#). In the multi-guide RNA, the concentration of each individual gRNA is equal.

38. Leave the RNP complex at room temperature for 10 min.
39. Place 3 mL of Buffer E into the disposable Neon Tube.
40. Select the following settings on the Neon device:

Neon device settings	
Category	Settings
Voltage	1,350 V
Width	2 ms
Pulses	2

Note: The above settings have been optimized for adult human intestinal organoids and may need to be optimized for other cell types.

41. Mix the cell suspension with the RNP complex; they should be in equal volumes if measured correctly.
42. Use the Neon pipette to aspirate 10 μ L of the suspension, place into the Neon Pipette Station and press "Start" on the Neon device interface to trigger the electroporation.

Note: The Neon pipette tip should be changed after 2 electroporation triggers, while the Neon Tube should be changed after 10 electroporation triggers.

43. Immediately after electroporation, transfer cells to a 1.5 mL microtube from step 31. Leave to rest at room temperature for 10 min.
44. Centrifuge at 400 $g \times 6$ min at 4°C.
45. Remove supernatant, and add 40 μ L/well of BME.
46. Plate on 24-well plate and leave in a 37°C incubator for 30 min.
47. Add 500 μ L of human IntestiCult to each well.
48. After 48 h, change media to human conditioned media. Refresh every 2–3 days.

Immunofluorescent staining of organoids

⌚ Timing: 2 days

Following electroporation, organoids should be allowed to recover and proliferate for 7–10 days, after which they may be passaged as normal. It is essential to validate that successful gene editing has occurred. This can be done via Sanger sequencing and Western blot, for which standard techniques apply. Another approach for validation is immunofluorescent staining, which is described in this section.

49. Prepare Organoid blocking solution as follows, and keep on ice:

Organoid blocking solution	
Reagent	Final concentration
DPBS	100 mM
BSA	3% (w/v)
Triton X-100	1% (v/v)
Saponin	1% (w/v)
Secondary Antibody Animal Serum	1% (v/v)

Note: It is important to make the Organoid staining solution fresh each time.

50. Fix organoids by adding 500 μ L of 2% (v/v) paraformaldehyde in 100 mM PBS to each well.
51. Incubate the cell culture at room temperature for 30 min.

⚠ CRITICAL: Perform in a chemical fume hood as fixative chemicals are toxic. Be careful not to come into contact with fumes. Never perform in a biosafety cabinet.

52. Prepare PBSB as follows, and keep on ice:

PBSB	
Reagent	Final concentration
DPBS	100 mM
BSA	0.1% (w/v)

Note: Transfer pipettes should always be coated with PBSB before use.

53. Using a transfer pipette, remove fixative and gently add ice-cold DPBS to each well.
54. Transfer fixed organoids into a 15 mL centrifuge tube, and add ice-cold DPBS to 10 mL. Invert the tube gently.

△ **CRITICAL:** Do not vortex or centrifuge as this may damage the organoids.

55. On ice, allow organoids to settle under gravity. This usually takes about 10 min.
56. Remove supernatant. Fill tubes with appropriate Organoid blocking solution and incubate with gentle agitation for 3 h at room temperature.
57. Allow organoids to settle under gravity for 10 min.
58. Prepare diluted mouse anti-FBXW7 antibody 1:100 in Organoid blocking solution.

Note: Antibody dilution concentrations need to be optimized for individual antibodies.

59. Remove supernatant and replace with diluted FBXW7 antibody.

△ **CRITICAL:** Samples must be protected from light and kept at 4°C from this step.

60. Incubate organoids with primary antibodies for 24 h with gentle agitation.
61. Prepare Organoid washing solution as follows, and keep on ice:

Organoid washing solution	
Reagent	Final concentration
DPBS	100 mM
BSA	3% (w/v)
Triton X-100	1% (v/v)
Saponin	1% (w/v)

62. Allow organoids to settle under gravity for 10 min.
63. Remove supernatant. Wash organoids with Organoid washing solution, inverting the tube carefully 10 times.
64. Repeat steps 62 and 63 another 3 times.

Note: It is very important to wash off primary antibodies as over exposure to primary antibodies will lead to nonspecific staining of sample and poor image quality.

65. Allow organoids to settle under gravity for 10 min.
66. Prepare diluted goat anti-mouse antibody at 1 µg/mL in Organoid washing solution.
67. Incubate organoids with secondary antibodies for 2 h with gentle agitation.
68. Repeat steps 62–64.

Note: Staining should remain visible under a fluorescence microscope for weeks.

Optional: Phalloidin staining. Incubate organoids for 30–60 min in DPBS with fluorescent phalloidin diluted 1:400, at room temperature with gentle agitation and light protection. Wash organoids twice with DPBS.

Optional: DAPI staining. Prepare DAPI to working concentration of 300 nM in DPBS. Incubate organoids in DAPI for 1–5 min at room temperature. Wash organoids twice with DPBS.

Note: Stain should remain visible for 1 week.

EXPECTED OUTCOMES

This protocol allows for the generation and validation of high-efficiency knockouts of any gene of interest, and can be performed within a short period of 2–3 weeks from the time a surgical biopsy specimen is obtained. Prior to the use of multi-guide RNA RNP electroporation, alternative approaches included the use of a lentivirus transduction system, which can often take 2–3 months to set-up, or the use of RNP electroporation with a sgRNA, which suffers from low knockout efficacy in human organoids.

On completion of this protocol, Synthego's Inference of CRISPR Edits (ICE) software (<https://ice.synthego.com/>) may be used to determine the knockout efficacy following CRISPR gene editing. Knockout efficacies from 75% – 100% have been generated using this protocol. Successful knockouts should be further validated with western blot (Figure 2), as well as with immunofluorescence (Figure 3) described here in this protocol. Once validated, knockouts may be used in downstream experiments as required.

LIMITATIONS

While this protocol generates a high knockout efficacy following CRISPR-Cas9 gene editing, the edited cells are not isogenic. Generating isogenic knockout clones requires single-cell cloning of the organoids, and has been described using FACS sorting.⁴ Nonetheless, the high knockout efficacy which can be obtained using the multi-guide RNP electroporation approach described here ensures that a greater percentage of successfully gene edited clones will be obtained following FACS sorting. Our protocol therefore serves as an adjunct which can be used in tandem with other FACS sorting protocols.

Another limitation of adult human intestinal organoids is their tendency to senesce after about eight passages. Consequently, organoids will need to be repeatedly generated, and gene edited. This could result in batch effects occurring between samples, and will need to be accounted for in downstream analyses.

TROUBLESHOOTING

Problem 1

Few crypts are viable from the biopsy specimen and do not form organoids.

Potential solution

In our experience, there is some variation in the organoid-forming capacity for different specimens. To optimize the number of crypts which can be harvested, the following steps can be undertaken. Minimize warm ischemia time by transporting the specimen on ice at all times. Ensure that all solutions are warmed to 37°C prior to use. All media should be newly generated and supplemented with Y27632. If this fails, obtain a new specimen.

Problem 2

Low knockout efficacy (<50%) on ICE analysis.

Potential solution

Cells need to be in a single-cell suspension at the time of RNP electroporation to improve efficacy. This can be aided by increasing the time in which the organoids are incubated in TrypLe at 37°C or by

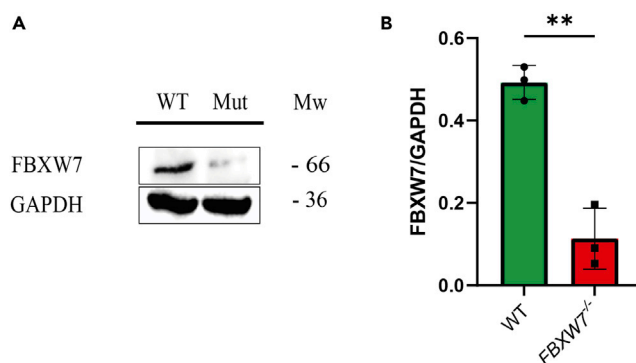


Figure 2. Western blot validation

(A) Western blot of FBXW7 protein showing knockout.

(B) FBXW7 protein expression relative to GAPDH (n = 3) (p = 0.0015).

tritulating organoids with P1000 pipette instead. Note however that overly aggressive dissolution of organoids can lead to increased cell death.

Another solution is to perform multi-guide CRISPR-Cas9 gene editing twice on the same cell sample. Following initial gene editing, if the efficacy is low, cells which have gone through one round of gene editing can be electroporated with the RNP complex again. Cells which have not been edited will continue to possess the guide RNA binding sites, which will allow for a second round of gene editing, therefore increasing efficacy even further. Note that this could lead to non-specific gene editing. Also, this cannot be repeated too many times as cells begin to lose their viability when subjected to multiple rounds of electroporation.

Problem 3

Poor organoid viability after CRISPR electroporation.

Potential solution

Ensure that Y27632 has been added to media as this reagent helps to prevent anoikis related cellular death. Organoids could also be subject to over trypsinization. To prevent this, reduce the time in which the organoids are incubated with TrypLe in the 37°C water bath.

Problem 4

High background signal in immunofluorescent imaging.

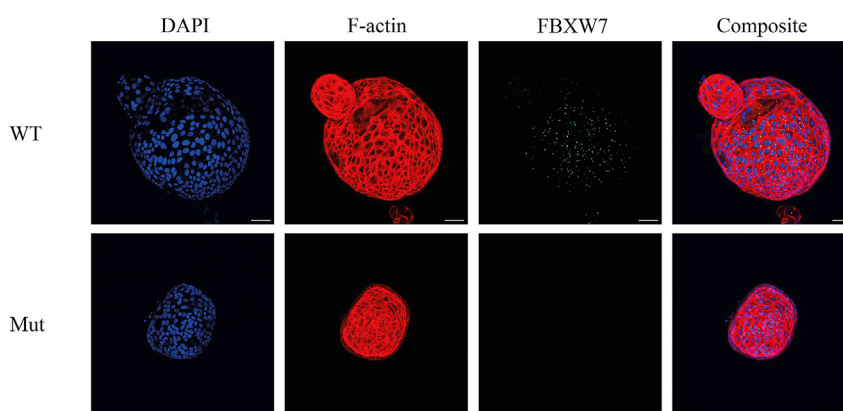


Figure 3. Immunofluorescent validation

Immunofluorescent validation of FBXW7 wild-type and knockout with DAPI (blue), F-actin (red) and FBXW7 (green). Scale bar equals 50 μ m.

Potential solution

Background signal can be caused by autofluorescence and nonspecific binding of antibodies. Produce two negative control samples that follow the same procedure – one without the primary and the other without the secondary antibodies. Adjust imaging analysis based on observed autofluorescence. Change antibody concentrations and incubation times based on the amount of nonspecific binding from the negative control samples.

Problem 5

No signal in Immunofluorescent imaging.

Potential solution

If there is no observable signal from immunofluorescent imaging, first check that the primary antibodies used react with the sample species, and the secondary antibodies react with the primary antibody species. Check that the antibodies were stored at the correct temperature and with light protection via the manufacturer's instructions. Otherwise, increase the concentration of the antibodies used and increase the antibody incubation time. Also, consider using a positive control cell line that is known to contain your target.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Prof. Simon James Alexander Buczacki (simon.buczacki@nds.ox.ac.uk).

Materials availability

This protocol did not generate new unique reagents.

Data and code availability

This study did not generate datasets or code.

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AUTHOR CONTRIBUTIONS

Conceptualization, D.K.H.C., S.J.A.B.; Investigation, D.K.H.C., S.D.C.; Writing – original draft, D.K.H.C.; Writing – review and editing, all authors; Supervision, S.J.A.B.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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