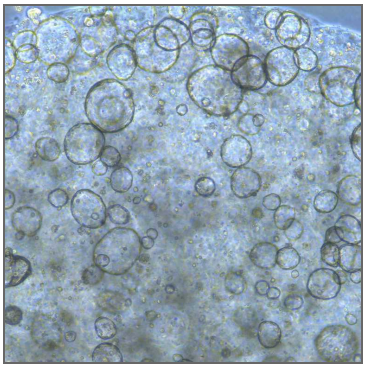

APR 15, 2024

Generating stably-expressing Cas9 cancer organoid lines

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DOI:
dx.doi.org/10.17504/protocols.io.6qpvr3ydovmk/v1

External link:
https://www.sanger.ac.uk/group/garnett-group/

Protocol Citation: Jade Smith, Tessa Fowler, Agnieszka Andres, Adam Jackson, Emily Souster, Hazel Rogers, Alexandra Beck, Charlotte Beaver, Mathew Garnett 2024. Generating stably-expressing Cas9 cancer organoid lines. **protocols.io** https://dx.doi.org/10.17504/protocols.io.6qpvr3ydovmk/v1

Protocol status: Working
We use this protocol and it's working

Created: Dec 29, 2023

Last Modified: Apr 15, 2024

PROTOCOL integer ID: 92810

Keywords: Cancer Organoids, CRISPR/Cas9, Antibiotic Titrations, Cas9 Transduction, Cas9 Activity Assay, Stably Expressing Cas9, Wellcome Sanger Institute, Organoids

ABSTRACT

This protocol aims to establish a robust Cas9 expression system in cancer organoids through a three-stage process. The first stage involves titrating blasticidin to determine the optimal concentration for eliminating wild-type cells while supporting the survival and growth of Cas9-expressing cells. The second step introduces the Cas9 gene, and the specified blasticidin concentration selects and maintains Cas9-positive cells. The final stage assesses Cas9 activity, ensuring functionality is above 75%. This established system facilitates precise and genome-wide gene modification using the 'lentiCas9-Blast' CRISPR-Cas9 guide RNA system.

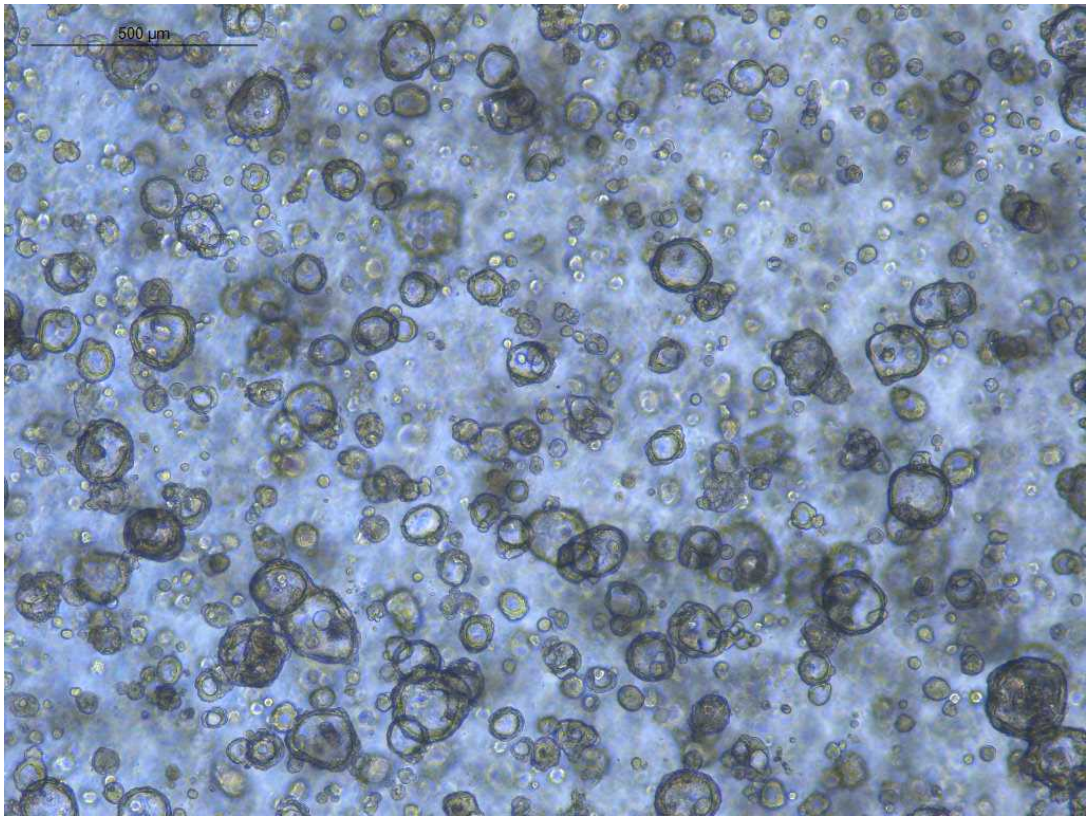
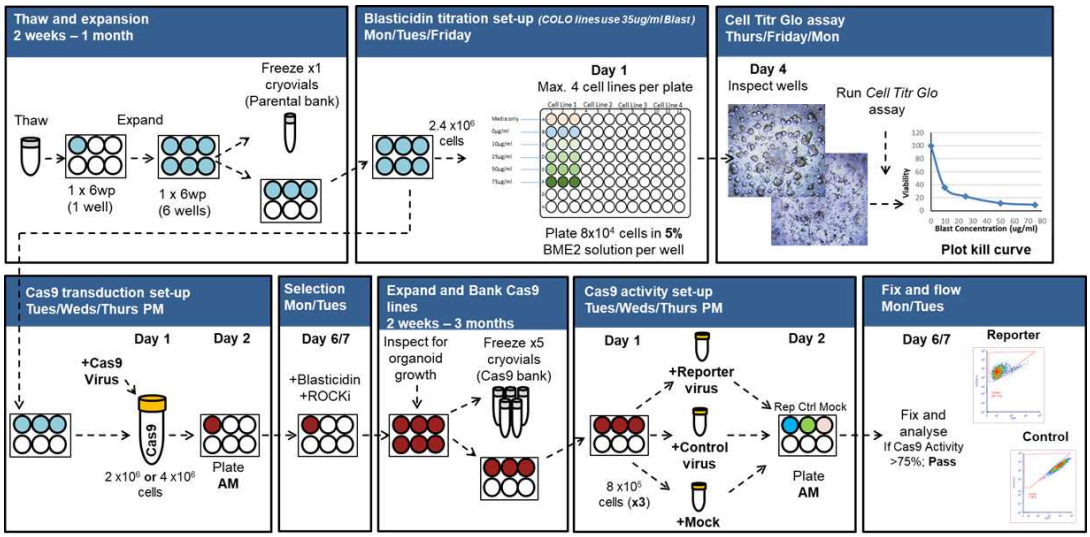


Image of a cancer organoid derived from a colon tumour sample

Process Diagram



Process Diagram

ATTACHMENTS

addgene-plasmid-52962-sequence-322376-map.png

PROTOCOL REFERENCES

Emily Souster, Verity Goodwin, Adam Jackson, Charlotte Beaver, Rizwan Ansari, Fiona Behan, Mathew Garnett 2020. Cas9 transduction of cancer cell lines. **protocols.io**https://dx.doi.org/10.17504/protocols.io.bg4ijyue

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Sanjana NE, Shalem O, Zhang F. Improved vectors and genome-wide libraries for CRISPR screening. Nat Methods. 2014 Aug;11(8):783-784. doi: 10.1038/nmeth.3047. PMID: 25075903; PMCID: PMC4486245.

GUIDELINES

- Ensure the cell suspension is mixed thoroughly at each step to create an even single-cell suspension before plating, using reservoirs and multi-channel pipettes where possible to minimise ergonomic strain and to maintain homogenous solutions throughout.
- Avoid prolonged maintenance of organoids in a single-cell state.
- Refrain from the freeze/thaw cycle of virus stocks.
- Prioritise using freshly prepared virus stocks to maintain optimal viral integrity.
- Ensure the correct concentration of virus is used in the experimental setup.
- Adequate dissociation is critical for efficient viral exposure and subsequent gene delivery.
- Be aware that some cell lines may exhibit lower uptake of viral particles; which may make them unsuitable for this process.
- Black-walled 96-well plates are used in this protocol, as we have found luminescence can carry over into neighbouring wells in clear plates.

MATERIALS

- 1X Dulbecco's Phosphate Buffered Saline (DPBS) **Thermo Fisher Scientific Catalog #**14190094
- TrypLE™ Express Enzyme (1X), no phenol red **Thermo Fisher Catalog #**12604021
- 10mg/ml Blasticidin **InvivoGen Catalog #**ant-bl-1
- 3.7% Formaldehyde **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**11-0705
- Cultrex Reduced Growth Factor Basement Membrane Extract, Type 2, Select **Bio-Techne Catalog #**3533-005-02
- CellTiter-Glo(R) 2.0 Assay **Promega Catalog #**G9243
- Y-27632 ROCK Inhibitor **Selleckchem Catalog #**S1049
- 10mg/ml Polybrene **Merck Millipore (EMD Millipore) Catalog #**TR-1003-G
- Trypsin-EDTA (0.25%) phenol red **Thermo Fisher Scientific Catalog #**25200072
- eBioscience™ Fixable Viability Dye eFluor™ 780 **Thermo Fisher Catalog #**65-0865-14
- Anti-Adherence Rinsing Solution **STEMCELL Technologies Inc. Catalog #**07010
- lentiCas9-Blast **addgene Catalog #**52962
- Black walled 96 well plate **Fisher Scientific Catalog #**10419822
- Costar® 6-well Clear TC-treated Multiple Well Plates, Individually Wrapped, Sterile **Corning Catalog #**3516
- Corning® 50 mL Mini Bioreactor **Corning Catalog #**431720
- Falcon Round bottomed 5ml tube with cell strainer lid **VWR International Catalog #**734-0001
- Cell scraper **Corning Catalog #**3011

Organoid specific culture media - Made to specification
Transduction media - Organoid specific culture media + Y-27632 ROCK Inhibitor (final concentration 2.5 micromolar (µM))
GFP/mCherry V4 (Control Vector) - Addgene 67981
BFP/GFP V4 (Control Vector) - Addgene 67979
BFP/GFP/gGFP V4 (Reporter Vector) - Addgene 67980
GFP/mCherry/gGFP V4 (Reporter vector) - Addgene 67982

Equipment

37 °C 5% CO_2 Incubator
Light microscope
Microbiological safety cabinet (CLASS II)
Pipette boy
Stripettes
Centrifuge
Pipettes and tips
Cell counter

SAFETY WARNINGS

- Lentiviral vectors can infect human cells. However, they are not able to replicate, so the pathogenicity is considered negligible and the risk is reduced by ensuring the correct use of PPE i.e. correct gloves, lab coat and eye protection.
- If centrifuge buckets are available they must be sealed using safety caps, which then must only be opened in a microbiological safety cabinet this is to prevent any biohazard aerosol exposure post-centrifugation.
- Many steps involve repetitive movements, ensure the use of multichannel and electronic pipettes where possible to reduce ergonomic stress.
- All lentiviral waste should be inactivated with recommended local waste routes.

BEFORE START INSTRUCTIONS

- If required, ensure that all media is pre-warmed before use.
- If required, Cell Titer-Glo 2.0 reagent is light-sensitive, so try to avoid exposure when using it.
- If needed, thaw an aliquot of polybrene.
- When needed, thaw an appropriate amount of Cas9 lentivirus for the number of transductions you will carry out.

Blasticidin titration

1 Day 1: Titration plate set up

Note

This assay is set up using previously expanded organoids.

1.1 Pre-warm organoid specific culture media to room temperature or place in a water bath at 37 °C for 00:10:00 10m

1.2 Aspirate media from well plates and add 2 mL TrypLE to each well.

1.3 Using a cell-scraper, detach BME2 drops containing the cancer organoids from the plate and transfer organoid suspension to an appropriately sized tube.

1.4 Pipette suspension up and down multiple times to dissociate organoids from the BME2.

1.5 Incubate at 37 °C 5% CO_2.

1.6 Check organoid suspension under the microscope every 00:15:00, to assess and monito 15m the dissociation of the organoids.

> **Note**
>
> Mix the cell suspension to help dissociate the organoids. Stop the incubation once the organoids have broken down to single cells.

1.7 Centrifuge at 800 x g for 00:02:00. 2m

1.8 Aspirate supernatant and resuspend in 5 mL organoid specific culture media.

1.9 Resuspend 2.4x10^6 cells in 2.7ml of organoid specific culture media + 300 µL BME2 (This will give a final seeding density of 8x10^4 cells per well once plated, Rows B-F of Fig.1).

Prepare a control stock solution containing organoid specific culture media with 5% BME2 (Row A of Fig.1).

> **Note**
>
> The titration is carried out in triplicate. Therefore, a 96-well plate can be used to titrate up to 4 cell lines at a time.

1.10 Make up enough volume to plate all required wells for running each required antibiotic concentration in triplicate (plus extra 10% for dead volume).

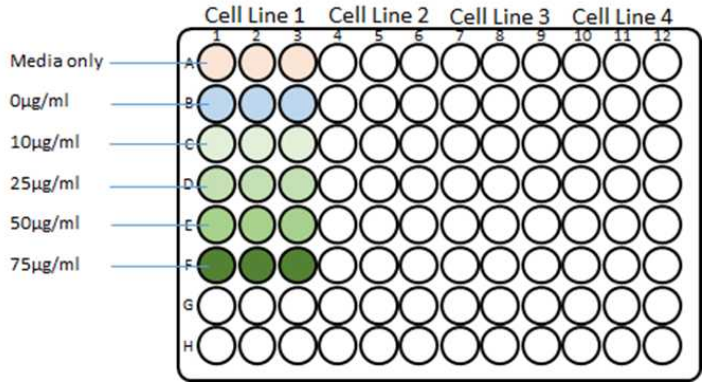

Fig.1: Layout of blasticidin titration plate

Note

Row A: Control (Step 1.13)
Row B-F: 100 µL of cells per well + 100 µL media containing blasticidin per well (Step 1.14).

1.11 Add 100 µL of the cell suspension containing BME2 to Rows B-F (according to Fig.1).

1.12 Incubate plate at 37 °C, 5% CO_2 for 00:10:00 to allow the BME2 to polymerise. 10m

1.13 Plate control wells (in triplicate) containing 200 µL organoid specific culture media containing 5% BME2 and no cells.

1.14 Make up blasticidin antibiotic solutions at 2x concentration in organoid specific culture media, containing no BME2 in 5 mL tubes.

Blasticidin dilutions		
Final concentration (once plated)	**10 mg/mL blasticidin stock (µL)**	**Media (mL)**
0 µg/mL	0	0.5
10 µg/mL	1	0.5
25 µg/mL	2.5	0.5
50 µg/mL	5	0.5
75 µg/mL	7.5	0.5

Fig.2: Table showing antibiotic concentrations

Safety information

Blasticidin is toxic if swallowed, and harmful if it comes into contact with skin.

1.15 Add 100 µL of the blasticidin antibiotic stock into the corresponding wells in Fig.1.

1.16 Incubate the plate for 72:00:00 at 37 °C, 5% CO2. 3d

2 Day 4: Assess cell viability using CellTiter-Glo 2.0 assay

2.1 Run a CellTiter-Glo 2.0 viability assay following the manufacturer's instructions.

celltiterglo-2-0-assay-protocol.pdf

Note

We dilute the reagent 1:5 rather than 1:2 with the cell suspension. It is recommended by the manufacturer to use white plates. However, we found the luciferase signal to be too strong, therefore we recommend using black plates instead.

2.2 Generate a kill curve graph using the data collected. The 'kill concentration' is the concentration of an antibiotic at which organoid growth is completely inhibited.

Row A is used as negative control to show background luminescence
Row B is the positive control to compare against wells without antibiotics

Expected result

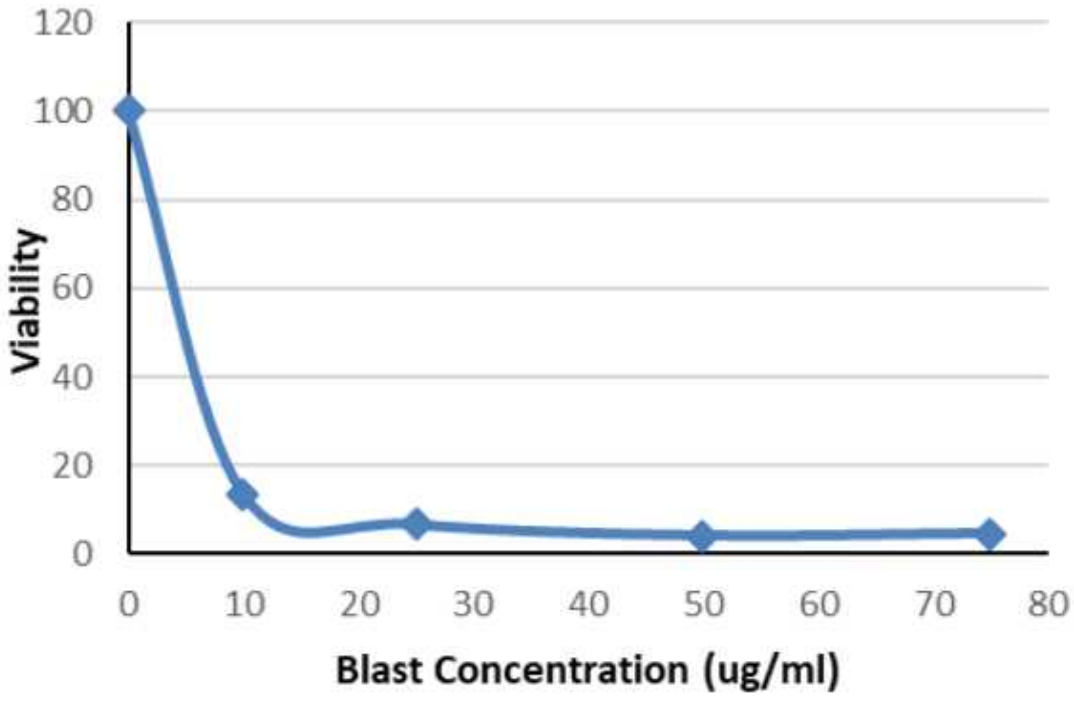

Fig.3: Kill concentration for this example organoid line is equal to 25 µg/ mL

Cas9 transduction of cancer organoids

34m

3 Day 1: Transduction setup

3.1 Prepare transduction media, add 5 µL ROCKi Y-27632 (10 mM) to 20 mL of organoid specific culture media (2.5 µM final concentration; dilution 1:4000).

3.2 Aspirate media from well plates and add 2 mL TrypLE to each well of a 6 well plate.

3.3 Using a cell-scraper, detach BME2 drops containing the cancer organoids from the plate and transfer organoid suspension to an appropriately sized tube.

3.4 Pipette suspension up and down multiple times to dissociate organoids from the BME2.

3.5 Incubate at 37 °C 5% CO_2.

3.6 Check organoid suspension under the microscope every 00:15:00, to assess and monito 15m the dissociation of the organoids.

Note

Mix the cell suspension to help dissociate the organoids. Stop the incubation once the organoids have broken down to single cells.

3.7 Centrifuge at 800 x g for 00:02:00 . 2m

3.8 Aspirate supernatant and resuspend in 10 mL of transduction media (more or less can be added depending on the size of the cell pellet).

3.9 Perform a cell count to calculate the total number of cells.

3.10 Prepare a preparation mix using the cell suspension and transduction media to achieve a final concentration of between $2x10^6$ and $4x10^6$ cells (with the minimum cells needed to perform a Cas9 transduction being $2x10^6$).

Note

We use $2x10^6$ (for good growing lines) or $4x10^6$ (for difficult lines) cells for Cas9 transduction.

3.11 

Please consult Fig.4 for the overall volumes, where the transduction media constitutes the remaining volume. Please consult Fig.4 for the overall volumes, where the transduction media constitutes the remaining volume. Add preparation mix, Cas9 transduction virus and polybrene into a 50 mL bioreactor tube using the table below.

Safety information

Lentiviral vectors can infect human cells. Ensure correct use of PPE to reduce the risk.

Note

Please refer to Fig. 4 for the total volumes, where the transduction media constitutes the remaining volume.

	A	B	C	D
	Cell count	**Amount of virus (mL)**	**Amount of polybrene (µL)**	Total volume (mL)
	$2x10^6$	1.5	5	3.5
	$3x10^6$	2.25	7.5	5.25
	$4x10^6$	3	10	7

Fig.4: Table showing transduction reagent volumes per required cell number. (Final concentration for polybrene is 10 µg ml^−1 and Y-27632 ROCKI 2.5 µM).

3.12 Incubate the 50 mL bioreactor tube prepared in step 3.11 Overnight at 37 °C, 5% CO_2.

4 Day 2: Plating cells

4.1 Centrifuge at 800 x g for 00:02:00. 2m

4.2 Aspirate the supernatant.

4.3 Seed 2x10^6 cells in 230 µL of 80% BME2. Seed as 15 µL drops in a one well of 6 well plate (230 µl per well).

4.4 Incubate at 37 °C for 00:15:00 then add 2 mLs of transduction media. 15m

4.5 Incubate cells at 37 °C, 5% CO2.

5 Day 6: Blasticidin selection

5.1 Prepare relevant organoid specific culture media with blasticidin (using the concentration based on the results obtained from the kill curve refer to Fig.3).

Safety information

Blasticidin is toxic if swallowed and harmful if it comes into contact with skin.

5.2 Replace media on plates or flasks with media containing blasticidin at [M] 25 mg/mL (obtained at Fig.3). For example: if the concentration is 25 µg/mL add 12.5 µl to 5 mL media ((volume x 2.5) / 1000 (to convert mL to µL)).

Note

From this point onwards, cells should be maintained in a medium containing antibiotics.

Full selection level is often not achieved until after the lines have been passaged. It is advised not to passage organoid lines at the point of selection despite density or confluence.

5.3 Expand until required number of cells for endpoint experiments has been reached (e.g. assessment of Cas9 activity).

Note

Organoids usually need one week post-passage to recover before further manipulation.

Assessment of Cas9 activity assay

44m

6 Day 1: Assay set up

6.1 Prepare transduction media, by adding 7.5 µL of ROCKi Y-27632 [M] 10 millimolar (mM) to 30 mL of organoid specific culture media.

Note

Cells need to remain in transduction media throughout this protocol.

The final concentration of ROCKi Y-27632 should be [M] 2.5 micromolar (µM) (dilution 1:4000).

6.2 Aspirate media from well plates and add 2 mL TrypLE to each well.

6.3 Using a cell-scraper, detach BME2 drops containing the cancer organoids from the plate and transfer organoid suspension to an appropriately sized tube.

6.4 Pipette suspension up and down multiple times to dissociate organoids from the BME2.

6.5 Incubate at 37 °C 5% CO_2.

6.6 Check organoid suspension under the microscope every 00:15:00, to assess and monito 15m the dissociation of the organoids.

> **Note**
>
> Mix the cell suspension to help dissociate the organoids. Stop the incubation once the organoids have broken down to single cells.

6.7 Centrifuge at 800 x g for 00:02:00. 2m

6.8 Aspirate supernatant and resuspend in 10 mL of transduction media (more or less can be added depending on the size of the cell pellet).

6.9 Perform a cell count to calculate the total number of cells.

6.10 Prepare a preparation mix using the cell suspension and transduction media to achieve a final concentration of 2.8x10^6 cells in 5.95 mL (equivalent to 8x10^5 cells in 1.7 mL accounting for dead volume) and polybrene.

6.11 Transfer 1.7 mL of the preparation mix into **3 x** 50 mL bioreactor tubes to include; **Mock, Control and Reporter** transductions with the final cell numbers, add 300 µL of each respective reagent to the tubes as shown in the table below.

Tube	Transduction	Number of cells	Volume of polybrene per tube (10µg/mL)	Cell suspension	Total volume	Vector
1	Mock	8x10^5	2 µL	1.7 mL	2 mL	300 µL media
2	Control	8x10^5	2 µL	1.7 mL	2 mL	300 µL BFP/GFP or mCh/GFP
3	Reporter	8x10^5	2 µL	1.7 mL	2 mL	300 µL BFP/GFP/gGFP or mCh/GFP/gGFP

Fig.5: Table showing reagents per 50 mL bioreactor tube.

6.12 Place bioreactor tubes in the incubator at 37 °C, 5% CO2 for Overnight incubation. 15m

7 Day 2: Plating cells

7.1 Transfer the **3 x** 50 mL bioreactor tubes to the centrifuge.

7.2 Centrifuge at 800 x g for 00:02:00 . 2m

7.3 Aspirate the supernatant for each bioreactor tube.

7.4 Resuspend the cells in each 230 µL of organoid specific culture media containing 80% BME2 (to account for pipetting loss) into each **Mock / Reporter / Control** tube.

7.5 Plate into 1 well of a 6 well plate for each **Mock / Reporter / Control**, dispensing small 15 µL droplets using a pipette.

7.6 Place plate in incubator at 37 °C , 5% CO2 for 00:10:00 until the BME droplets solidify. 10m

7.7 Add 2 mL of transduction media to each well.

Note

At this stage, the plated cells should need no further intervention until Day 6 when they are to be harvested for flow cytometry. However, it is best practice to keep checking the organoids and culture as required.

Formaldehyde fixation of organoids

36m

8 Day 6: Fixing and staining organoids for flow cytometry analysis

8.1 Prepare Live/Dead stain solution or antibodies.

> **Note**
>
> This protocol uses an e780 viability dye. For this reagent prepare a 1:10,000 dilution of e780 dye in PBS. Mix well and store at 4 °C (Solution can be used for 1 week from the time it was prepared).

8.2 Aspirate media from wells and resuspend in 1 mL of Trypsin-EDTA (0.25%) in a 2 mL tube.

8.3 Incubate for 00:15:00, until organoids have broken down to single cells. 15m

> **Note**
>
> Mix the solution every few minutes during the incubation. Some lines take longer to dissociate so do not leave any longer than 30 minutes.

8.4 Once organoids have broken down to single-cells stop the reaction by adding 1 mL (diluting 1:1) in media containing serum.

8.5 Centrifuge at 800 x g for 00:02:00. 2m

8.6 Aspirate supernatant and resuspend pellets in 200 µL Live/Dead dye solution (or specific antibody of choice).

8.7 For the Live/Dead solution, incubate at room temperature for 00:05:00 . (Follow specific guidelines for your antibodies). 5m

8.8 Add 1.8 mL of PBS (1:10 dilution).

8.9 Centrifuge at 800 x g for 00:02:00 . 2m

8.10 Aspirate supernatant and resuspend in 500 µL of 3.7% formaldehyde. Mix well by pipetting to ensure cells are fixed as single cells.

Safety information

3.7% formaldehyde must be prepared and used only in the chemical fume hood, using chemical resistant gloves. Waste must be kept in the fume hood and disposed of via the recommended route.

8.11 Incubate at 4 °C for 00:10:00 . 10m

8.12 Centrifuge at 800 x g for 00:02:00 . 2m

8.13 Carefully aspirate supernatant (in chemical fume hood).

Note

Cell pellets may become transparent and therefore difficult to see. It may also be sticky so can easily stick to pipette tips.

8.14 Resuspend the pellet in 500 µL (dependant on pellet size) PBS or alternative FACs buffer, and store it at 4 °C until ready for analysis by flow cytometry.

Note

Here we are measuring the expression of fluorescent proteins (in this case mCherry and GFP) using a flow cytometer. A reporter virus and control virus is used as guide to detect the presence of mCherry and the absence of GFP in the same cell population indicating successful Cas9-mediated gene editing (Refer to Fig.6). The mock is used as the negative control to help gate the un-transduced cells during FACS.

Expected result

If the cell line has >75% Cas9 activity, the line has passed our Cas9 activity assessment.

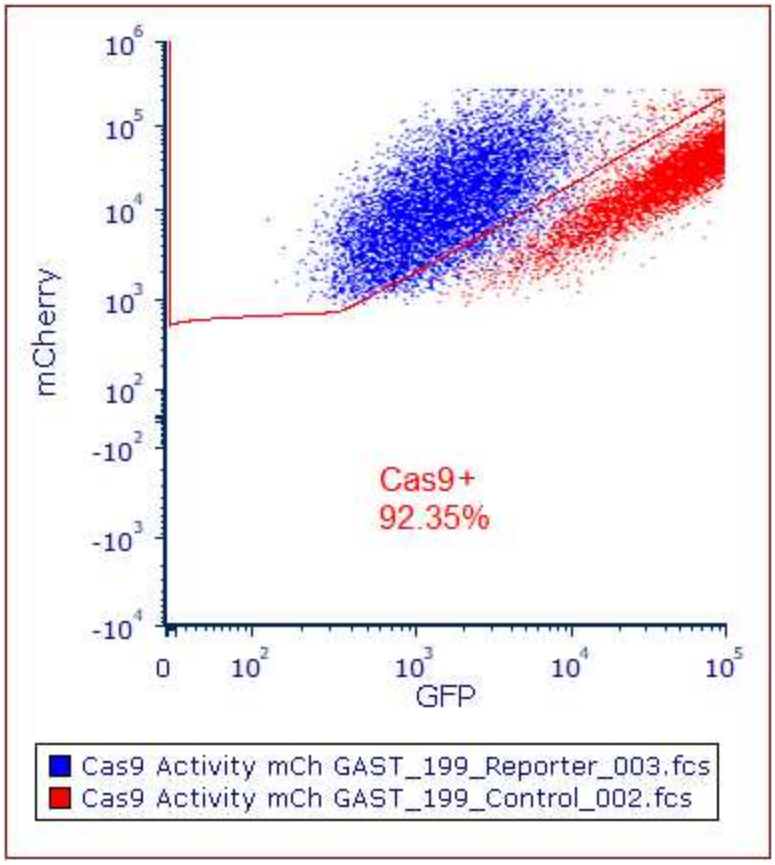

Fig. 6: Flow plot showing the presence of mCherry and GFP in the control and suppression of GFP expression in the reporter.