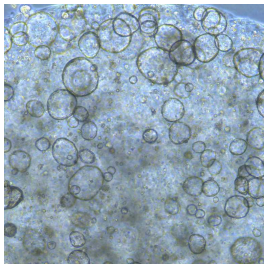
Nov 12, 2024 Version 2

Generating stably-expressing Cas9 cancer organoid lines V.2



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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.

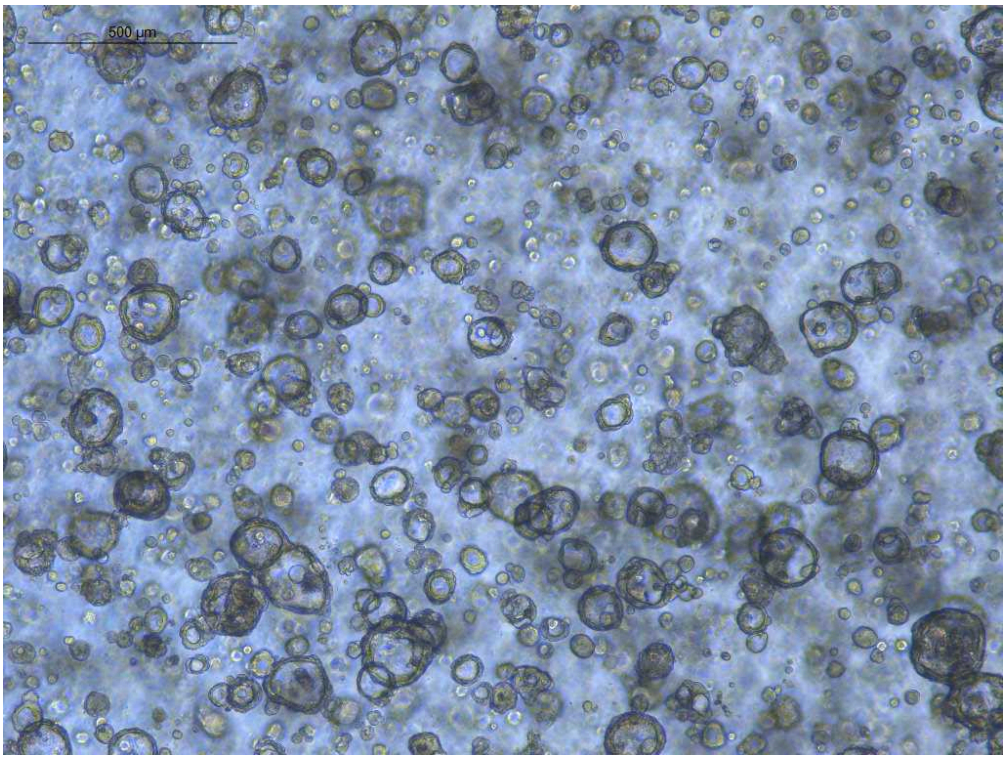
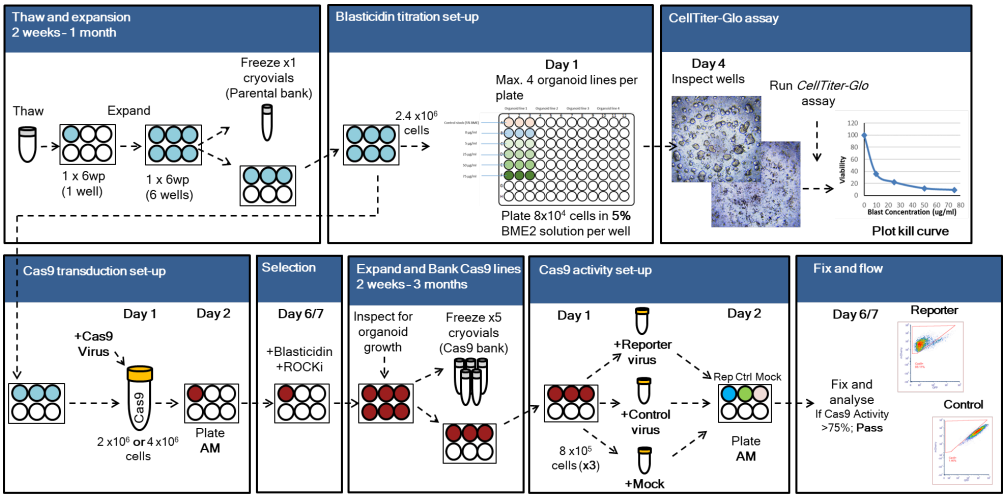


Image of a cancer organoid derived from a colon tumour sample

Process Diagram



Attachments

PNG

addgene-plasmid-5296...

238KB

Guidelines

- Thoroughly mix the cell suspension at each step to achieve an even single-cell distribution before plating, utilising reservoirs and multi-channel pipettes to reduce ergonomic strain and ensure homogenous solutions.

- Avoid maintaining organoids in a single-cell state for extended periods, and refrain from freeze/thaw cycles of virus stocks. Always use freshly prepared virus stocks to ensure optimal viral integrity and confirm that the virus concentration is appropriate for the experimental setup.

- Adequate dissociation is crucial for effective viral exposure and gene delivery. Be aware that some organoid lines may exhibit lower uptake of viral particles, which could render them unsuitable for this process.

- If required, Cell Titer-Glo 2.0 reagent is light-sensitive, so try to avoid exposure when using it.

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 (Avantor) 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

Chemical	Hazards	Hazard pictogram
Virkon	1% Virkon is harmful if swallowed, in contact with skin or if inhaled. Causes skin irritation, serious eye damage. May cause respiratory irritation. May produce an allergic reaction. Harmful to aquatic life with long lasting effects.	
Ethanol	Highly flammable liquid and vapour	
Formaldehyde	Toxic if swallowed, in contact with skin or if inhaled. Causes severe skin burns and eye damage. May cause an allergic skin reaction. May cause respiratory irritation. Suspected of causing genetic defects. May cause cancer. Causes damage to organs.	
Y-27632 (ROCKi)	Harmful if swallowed, in contact with skin or if inhaled.	
Trypsin-EDTA (0.25%)	May cause allergy or asthma symptoms or breathing difficulties if inhaled.	
Puromycin	Toxic if swallowed, harmful in contact with skin	
CellTiter glo 2.0	Harmful to aquatic life with long lasting effects	

- 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.
- It is recommended to use centrifuge buckets safety caps and to ensure the caps are only be opened in a microbiological safety cabinet 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 and disposed of via recommended local waste routes.

Before start

- If required, ensure that all media is pre-warmed to room temperature before use.
- 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.

1.2 Aspirate media from each well of the organoid culture plate 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 the 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 the suspension under a microscope every 00:15:00, to assess and monitor the dissociation of the organoids. 15m

> Note
>
> Mix the suspension thoroughly prior to each check to help dissociate the organoids. Stop the incubation once the organoids have broken down to single cells (It is recommended to not exceed 01:00:00 in TrypLE).

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

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

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

1.10 Resuspend 2.4×10^6 cells in 2.7 mL of organoid specific culture media + 300 µL BME2 This will give a final seeding density of 8×10^4 cells per well once plated (Rows B-F of Fig 1).

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

1.12 Set up the titration plate as detailed in Fig 1 below.

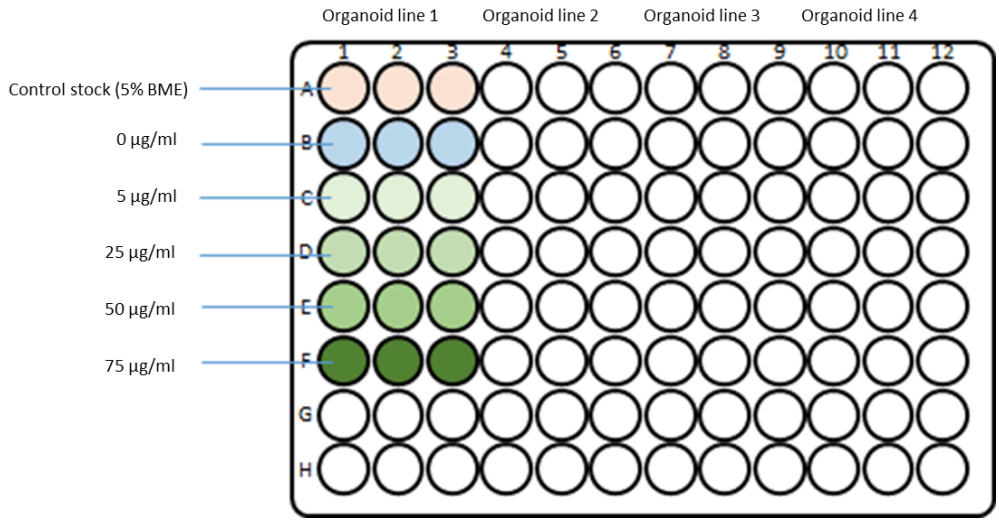


Fig 1: Layout of blasticidin titration plate

Row A = 200 µL control stock solution per well (step 1.11)
Rows B - F = 100 µL cell suspension (step 1.10)

Note

- Always seed 3 wells per row as the titration is carried out in triplicate.
- A 96-well plate can be used to titrate up to 4 organoid lines at a time.

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

1.14 Using a 10 mg/mL stock, prepare blasticidin antibiotic solutions at 2x concentrations in organoid specific culture media in tubes as outlined below in table 1.

Row (Fig.1)	2 x blasticidin concentration (µg/mL)	Volume 10 mg/mL blasticidin (µL)	Media volume (µL)	Total volume (µL)	Final plated blasticidin concentration (µg/mL)
B	0	0	2500	2500	0
C	20	5	2495	2500	10
D	50	12.5	2487.5	2500	25
E	100	25	2475	2500	50
F	150	37.5	2462.5	2500	75

Table 1: 2 x blasticidin concentrations using 10 mg/mL stock.

Note

- Prepare a minimum of 2.5 mL of each 2x antibiotic so that the volume is adequate for loading a multi-channel pipette without bubbles.
- Antibiotic dilutions should be prepared fresh on the day and kept at 4 °C until required.

Safety information

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

1.15 Remove the plate from the incubator and pipette 100 µL of the relevant 2x blasticidin stock into each well (Rows B-F) to achieve the final required blasticidin concentration, according to the plate layout 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 Thaw CellTiter-Glo 2.0 reagent and equilibrate to room temperature prior to use.

Safety information

CellTiter-Glo is harmful to aquatic life with long lasting effects.

Note

- CellTiter-Glo reagent can be stored at -20 °C and is stable for up to 4 freeze-thaws; thawed reagent can be kept at 4 °C for up to 5 months.
- CellTiter-Glo is light-sensitive so should be kept covered, and used in a cell culture hood with the light off where possible.

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

celltiterglo-2-0-assay-protocol.pdf

Note

This process dilutes the reagent 1:5 rather than 1:2 with the cell suspension. It is recommended by the manufacturer to use white plates. However, the luciferase signal was found to be too strong so using black plates is recommended.

2.3 Using the luminescence data, plot a kill curve to ascertain the lowest concentration of blasticidin which results in approximately 100% cell death after 72 hours. (Fig 2).

Note

To create the kill curve;
- Average the triplicate luminescence values to get a single value for each condition.
- Subtract the average background luminescence (Row A, media only) from the other averaged conditions (Rows B-F).
- Divide the average luminescence minus background for each concentration 10, 25, 50 and 75 µg/mL by the 0 µg/mL average to obtain a relative percentage viability.
- Plot these percentage viabilities on a graph against the blasticidin concentration.

Expected result

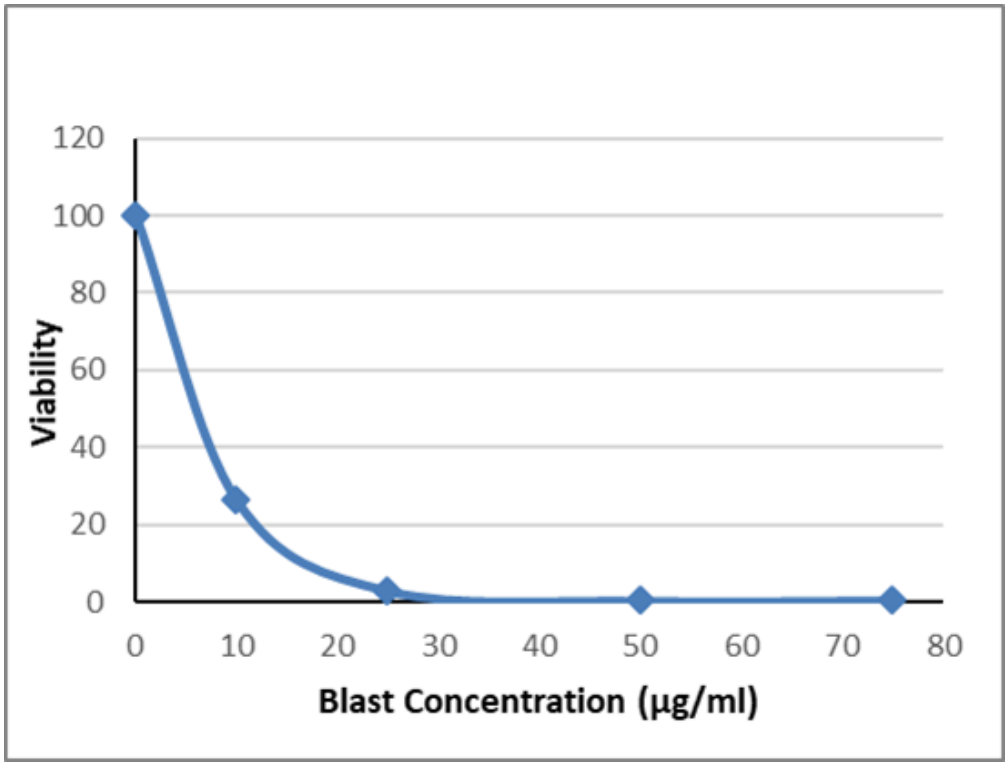


Fig 2: Kill concentration for this example organoid line is equal to 25 µg/mL

Cas9 transduction of cancer organoids

34m

3 **Day 1: Transduction setup**

Note

This transduction is set up using previously expanded organoids.

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 each well of the organoid culture plate and add 2 mL TrypLE to each well.

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 the suspension under a microscope every 00:15:00, to assess and monitor the dissociation of the organoids. 15m

> Note
>
> Mix the cell suspension to help dissociate the organoids. Stop the incubation once the organoids have broken down to single cells (It is recommended to not exceed 01:00:00 in TrypLE).

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 Make a preparation mix by combining the cell suspension with transduction media to achieve a final cell concentration 5.7×10^5 cells/mL.

Note

For Cas9 transduction, use 2x10^6 (for good growing lines) and up to 4x10^6 cells (for challenging lines).

3.11 Add preparation mix, Cas9 transduction virus and polybrene into a 50 mL bioreactor tube (Table 2).

Safety information

Lentiviral vectors can infect human cells. Ensure correct use of PPE and utilise recommended waste routes to reduce the risk.

To reduce the risk of aerosols, it is recommended to use centrifuge buckets that are sealed using safety caps and only opened in a microbiological safety cabinet.

Cell count (x10^6)	Amount of virus (µL)	Amount of polybrene (µL)	Volume of transduction media (µL)	Total volume (µL)
2	1500	5	2000	3500
3	2250	7.5	3000	5250
4	3000	10	4000	7000

Table 2: Table showing transduction reagent volumes per required cell number. (Final concentration for polybrene is 10 µg/mL 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 Pre-warm transduction media to room temperature.

4.2 Centrifuge the 50 mL bioreactor tube prepared in step 3.12 800 x g for 00:02:00 2m

> **Safety information**
>
> Lentiviral vectors can infect human cells. Ensure correct use of PPE and utilise recommended waste routes to reduce the risk.
>
> To reduce the risk of aerosols, it is recommended to use centrifuge buckets that are sealed using safety caps and only opened in a microbiological safety cabinet.

4.3 Aspirate the supernatant.

4.4 Resuspend 2×10^6 cells in 230 µL of 80% BME2 and seed as 15 µL drops into one well of 6-well plate (230 µL per well).

> **Note**
>
> If transducing more cells, adjust the volume to distribute the cells across 1 to 2 wells as needed.

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

4.6 Incubate cells at 37 °C, 5% CO2.

5 Day 6: Blasticidin selection

5.1 Aspirate the 2 mL transduction media and replace it with fresh media. Then, add blasticidin at the previously calculated concentration go to step #2.3.

Safety information

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

Note

From this point onwards, organoids should be maintained in a medium containing blasticidin.

Full selection 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.

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

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**

Note

This assay is set up using previously expanded Cas9 transduced organoids from go to step #5.2

Since this is an endpoint assay, blasticidin is not needed for the main set up. However, ensure that cancer organoids are cultured in parallel in a medium containing antibiotics for downstream analysis.

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 from [M] 10 millimolar (mM) stock).

6.2 Remove plate from incubator and 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 monitor the dissociation of the organoids. 15m

> Note
>
> Mix the cell suspension to help dissociate the organoids. Stop the incubation once the organoids have broken down to single cells (It is recommended to not exceed 01:00:00 in TrypLE).

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 Make a preparation mix by combining the cell suspension with transduction media to achieve a final concentration of 2.8×10^6 cells in a total volume of 5.95 mL (equivalent to 8×10^5 cells in 1.7 mL accounting for dead volume).

6.11 Add 7 µL of polybrene at a concentration of [M] 10 µg/µL to the mix.

6.12 Transfer 1.7 mL of the preparation mix into **3 x** 50 mL bioreactor tubes to include; **Mock, Control and Reporter** transductions.

6.13 Add 300 µL of each respective vector to the tubes (as shown in table 3 below).

Tube	Transduction	Volume of polybrene (10 µL/mL)	Total volume (µL)	Volume of vector (µL)	Vector
1	Mock	2	1700	300	Transduction media only
2	Control	2	1700	300	BFP/GFP or mCh/GFP
3	Reporter	2	1700	300	BFP/GFP/gGFP or mCh/GFP/gGFP

Table 3: Table showing reagents per 50 mL bioreactor tube.

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

7 Day 2: Plating cells

7.1 Pre-warm transduction media to room temperature.

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

7.3 Centrifuge at 800 x g for 00:02:00 2m

Safety information

Lentiviral vectors can infect human cells. Ensure correct use of PPE and utilise recommended waste routes to reduce the risk.

To reduce the risk of aerosols, it is recommended to use centrifuge buckets that are sealed using safety caps and only opened in a microbiological safety cabinet.

7.4 Aspirate the supernatant from each bioreactor tube.

7.5 Resuspend each pellet **Mock / Reporter / Control** in 230 µL of organoid specific culture media containing 80% BME2.

7.6 Plate 230 µL into 1 well of a 6 well plate for each **Mock / Reporter / Control**, dispensing small 15 µL droplets using a pipette.

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

7.8 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 organoid 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

Other antibodies can be used but 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 (The solution can be used for 1 week from the time it was prepared).

8.2 Aspirate media from wells and, using a cell-scraper, detach BME2 drops containing the cancer organoids and resuspend in 1 mL of Trypsin-EDTA (0.25%) in an appropriately sized tube.

Safety information

Trypsin-EDTA may cause allergic reactions, asthma symptoms, or breathing difficulties if inhaled.

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. Do not leave any longer than 00:30:00.

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

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

8.6 Aspirate supernatant and resuspend the pellets in 200 µL e780 dye solution.

8.7 Incubate at room temperature for 00:05:00 . 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

In this experiment, expression of fluorescent proteins (mCherry and GFP) is assessed via flow cytometry. A reporter virus and control virus serve as guides to identify cells showing mCherry expression and absence of GFP concurrently within the same cell population, which signifies effective Cas9-mediated gene editing (Fig 3).

Additionally, a mock control (no virus) is employed as a negative control. This control is used to accurately gate the cell populations of interest and distinguish between edited and non-edited cells based on fluorescence levels.

The loss of GFP in the reporter reflects successful Cas9 mediated gene editing, as it indicates disruption or knockout of the targeted gene. Therefore the percentage loss of GFP indicates the level of Cas9 activity.

Expected result

If the cell line exhibits over 75% Cas9 activity, it meets the criteria for passing our Cas9 activity assessment.

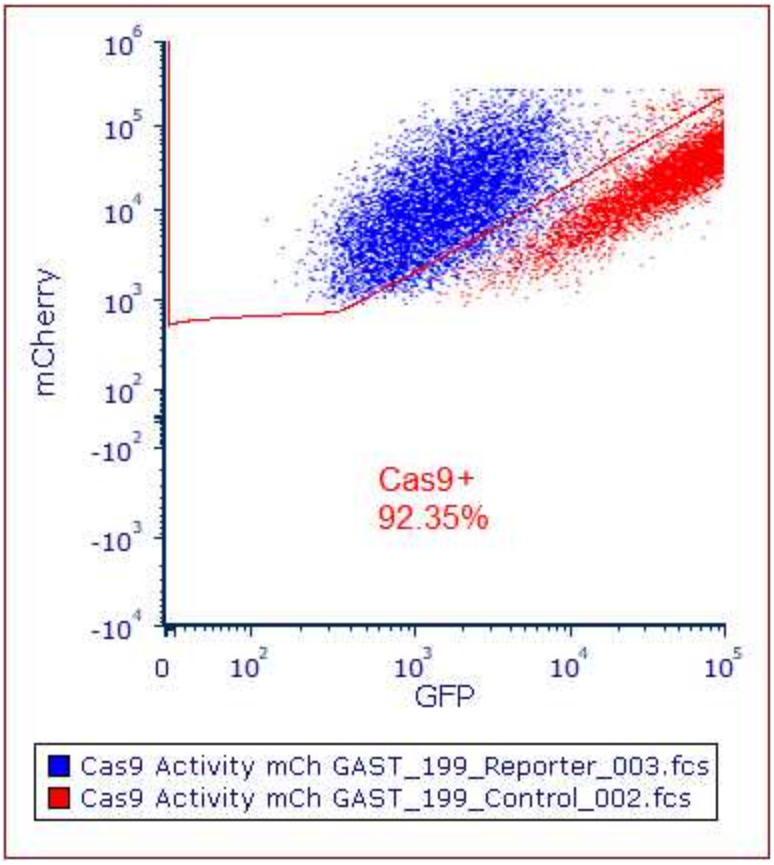


Fig 3: Flow cytometry plot displaying the presence of mCherry in the control population (Blue) group, along with an overlay illustrating the suppression of GFP expression in the reporter(Red).

Protocol references

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