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Whole-genome CRISPR Screening of stably expressing Cas9 Cancer Organoid Lines V.1

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We use this protocol and it's working

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Abstract

This protocol is for whole-genome CRISPR screening of stably expressing Cas9 cancer organoid lines in triplicate using the commercially available minimal genome-wide human CRISPR Cas9 library. The protocol uses lentiviral transduction as a method for gRNA delivery. This method can be adapted for other gRNA libraries.

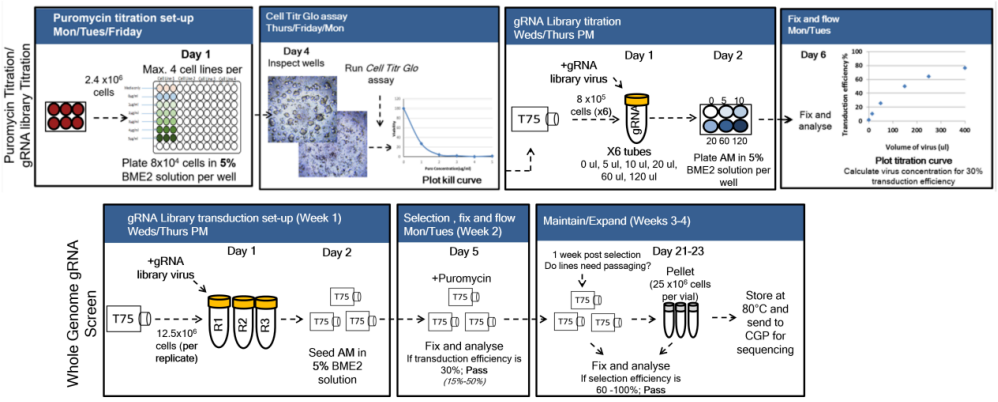
The protocol can be followed assuming the following is known:
- The number of days required for screening
- The size of the gRNA library
- The required coverage of the library

To allow for efficient scale up of the organoid culture for screen, a 5% suspension culture method can be used. This is not essential to be able to perform whole-genome CRISPR screening of stably expressing Cas9 cancer organoid lines, but provides a more scalable, ergonomic and cost efficient culturing method.

Prior to commencing the screen a puromycin antibiotic titration is used to identify the most suitable puromycin concentration for the selection of Cas9 positive cancer organoid lines transduced with gRNA library virus.

A gRNA library titration is then performed to determine the volume of library virus required to transduce Cas9 cancer cells at 30% transduction efficiency which is calculated using FACS analysis. This is to avoid host cells taking up more than one gRNA copy per cell, therefore an MOI of 30% should be aimed for.

Process Diagram



Process diagram outlining key steps for CRISPR-Cas9 screening in organoids.

This protocol uses a 21-23 day screen process.

Guidelines

It is important that all stages are carried out using the stably expressing Cas9 version of each cancer organoid line.

Ensure the cell suspension is mixed thoroughly at all stages to create an even single cell suspension before plating.

Avoid maintenance of organoids in a single-cell state for prolonged periods to retain viability.

Avoid freeze/thaw cycles of lentivirus stocks. Prioritising freshly prepared virus stocks will ensure viral integrity is maintained.

Puromycin titration;

It is advised to use black 96-well plates in this protocol, as luminescence can carry over into surrounding wells in clear plates.

All steps involved in the plate set up, including seeding cells, media, antibiotics and CellTiter-Glo should be carried out using reservoirs and multi-channel pipettes where possible to reduce ergonomic strain and to maintain homogeneous solutions throughout.

gRNA library titration;

The volumes of library titrated may differ depending on the batch of lentivirus or library used. To achieve a 30% MOI there may therefore be a requirement to dilute the virus prior to titrating. For this reason, an increased number of titration points may also be required. This may require some R&D to determine optimal volumes to titrate.

gRNA library transduction and screen;

The gRNA library transduction is carried out in triplicate with 12.5 ×10^6 cells seeded per replicate. Expansion of the line to a minimum of 40 ×10^6 cells is required for this process.

Following selection at Day 6 the organoid suspension should remain in puromycin selection media for the remainder of the screen.

Unless otherwise stated, all steps should be performed under sterile conditions in a microbiological safety cabinet.

Materials

- TrypLE™ Express Enzyme (1X), no phenol red **Thermo Fisher Catalog #**12604021
- 1X Dulbecco's Phosphate Buffered Saline (DPBS) **Thermo Fisher Scientific Catalog #**14190094
- 10mg/ml Puromycin **InvivoGen Catalog #**ant-pr-1
- CellTiter-Glo(R) 2.0 Assay **Promega Catalog #**G9243
- Black walled 96 well plate **Fisher Scientific Catalog #**10419822
- 37% Formaldehyde **AppliChem Catalog #**A0823
- eBioscience™ Fixable Viability Dye eFluor™ 780 **Thermo Fisher Catalog #**65-0865-18
- 10mg/ml Polybrene **Merck Millipore (EMD Millipore) Catalog #**TR-1003-G
- Costar® 6-well Clear TC-treated Multiple Well Plates Individually Wrapped Sterile **Corning Catalog #**3516
- Falcon Round bottomed 5ml tube with cell strainer lid **VWR International (Avantor) Catalog #**734-0001
- Corning® 50ml mini bioreactor **Corning Catalog #**431720
- Trypsin-EDTA (0.25%) phenol red **Thermo Fisher Scientific Catalog #**25200072
- Falcon™ 15mL Conical Centrifuge Tubes **Fisher Scientific Catalog #**14-959-53A
- Y-27632 ROCK Inhibitor **Selleckchem Catalog #**S1049
- Corning® 125 mL Polycarbonate Erlenmeyer Flask with Vent Cap **Corning Catalog #**431143
- Corning® 250 mL Polycarbonate Erlenmeyer Flask with Vent Cap **Corning Catalog #**431144
- Microcentrifuge tube Safe-Lock write-on 1.5mL Eppendorf Tube **Eppendorf Catalog #** 0030 120.086
- Ultra-low attachment 6-well plate **Corning Catalog #**CLS3471
- Corning® Ultra-Low Attachment 75cm² U-Flask Canted Neck Cell Culture Flask with Vent Cap **Corning Catalog #**3814
- Falcon 50mL Conical Polypropylene Centrifuge Tube with Flat Screw Cap, Sterile **Scientific Laboratory Supplies Ltd Catalog #**352070
- Cultrex Reduced Growth Factor Basement Membrane Extract, Type 2 **Bio-Techne Catalog #**3533-005-02
- MinLibCas9 Library† **addgene Catalog #**164896
- Corning® Cell Recovery Solution **Corning Catalog #**CLS354253

Organoid specific culture media- Made to specification
Transduction media - Organoid specific culture media + Y-27632 ROCK Inhibitor (final concentration 2.5 micromolar (µM))

Equipment

- 37 °C 5% CO_2 Incubator

Light microscope
Microbiological safety cabinet (CLASS II)
Centrifuge
Cell counter
Chemical fume hood
Plate reader
Pipette boy
Stripette
Pipettes and tips
Multichannel pipette and tips

Troubleshooting

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	

- Lentiviruses used in this protocol can infect human cells but are non-replicating and therefore the pathogenicity of these viruses is negligible. Correct use of PPE will reduce the risks.
- All lentiviral waste should be inactivated and disposed of using recommended local waste routes.

Before start

- Prior to each process, pre-warm culture media to room-temperature.
- Where required, prepare an aliquot of 1mg/ml puromycin (working concentration) by diluting a 10mg/ml stock 1:10 with sterile water.
- On Day 4 of the puromycin titration process, bring Cell Titer-Glo 2.0 reagent to room temperature. (It is light-sensitive and so it is advisable to keep the reagent covered at all times to avoid exposure to light when using).
- Prior to gRNA library transduction, thaw an aliquot of (10mg/ml) polybrene
- Where required, thaw the required volume of lentivirus for each process stage.
- When fixing organoids, dilute 37% formaldehyde solution 1:10 with DPBS and store at 4 °C

Puromycin titration

3d 0h 22m

1 **Day 1: Titration plate set up**

> Note
>
> This assay is set up using previously expanded organoids which are stably expressing Cas9.

1.1 Pre-warm organoid specific culture media to room temperature.

1.2 Aspirate media from each well of the organoid culture plate 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 the 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 organoid suspension under the microscope every 15 minutes to assess and monitor the dissociation of the organoids. Mix the suspension thoroughly prior to each check to help dissociate the organoids.

> Note
>
> Generally the suspension becomes cloudy once the majority of organoids are dissociated to small clumps of cells. 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 the supernatant and resuspend the pellet in an 10 ml of organoid specific culture media.

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

> Note
>
> A minimum of 12.5 x10^6 cells are required at this stage to ensure enough cells remain for transfer to 5% BME2 suspension culture (step 1.17).

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-G 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;

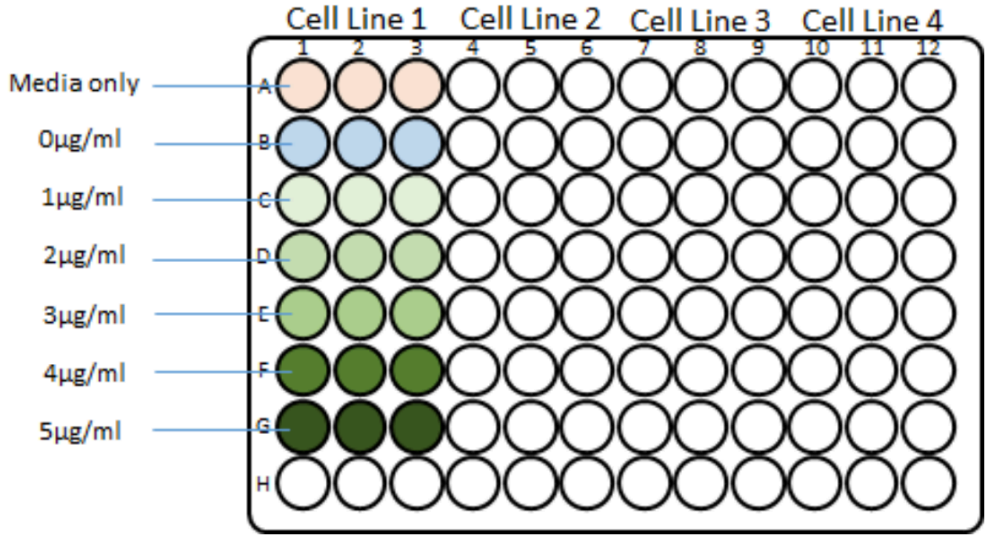


Fig 1: Puromycin titration plate set up
Row A = 200 µl control stock solution per well (step 1.11)
Rows B - G = 100 µl cell suspension (step 1.10)

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 cell lines at a time.

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

1.14 Using a 1 mg/ml stock, prepare puromycin antibiotic solutions at 2x concentrations in organoid specific culture media in 5 ml tubes as outlined below. (Table 1).

Safety information

Puromycin is toxic if swallowed and harmful in contact with skin.

Row (Fig 1)	2 x puromycin concentration (µg/ml)	Volume 1mg/ml puromycin (µl)	Media volume (ml)	Total volume (ml)	Final plated puromycin concentration (µg/ml)
B	0	0	2.50	2.5	0
C	2	5	2.45	2.5	1
D	4	10	2.40	2.5	2
E	6	15	2.35	2.5	3
F	8	20	2.30	2.5	4
G	10	25	2.25	2.5	5

Table 1: 2x puromycin concentrations using 1mg/ml puromycin 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 that they are required.

1.15 Remove the plate from the incubator and pipette 100 µL of the relevant 2x puromycin stock into each well (Rows B-G) to achieve the final require puromycin concentration according to the plate layout in Fig 1.

1.16 Incubate the plate for 72:00:00 at 37 °C 5% CO_2. 3d

1.17 Plate any remaining cells from step 1.10 into a 5% BME2 suspension culture by seeding cells into a solution of 19 mL organoid specific culture media + 1 mL BME2 using an ultra-low attachment T75 flask.

Note

A minimum of $10x10^6$ cells are required at this stage.

1.18 Incubate the ultra-low attachment T75 flask at 37 °C 5% CO_2.

Note

The seeded ultra-low attachment T75 flask is to be used for gRNA library titration set up (see section 3).

2 Day 4: Assessing cell viability using CellTiter-Glo

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 puromycin 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-G).
- Divide the average luminescence minus background for each concentration 1, 2, 3, 4 and 5 µg/ml by the 0 µg/ml average to obtain a relative percentage viability.
- Plot these percentage viabilities on a graph.

Expected result

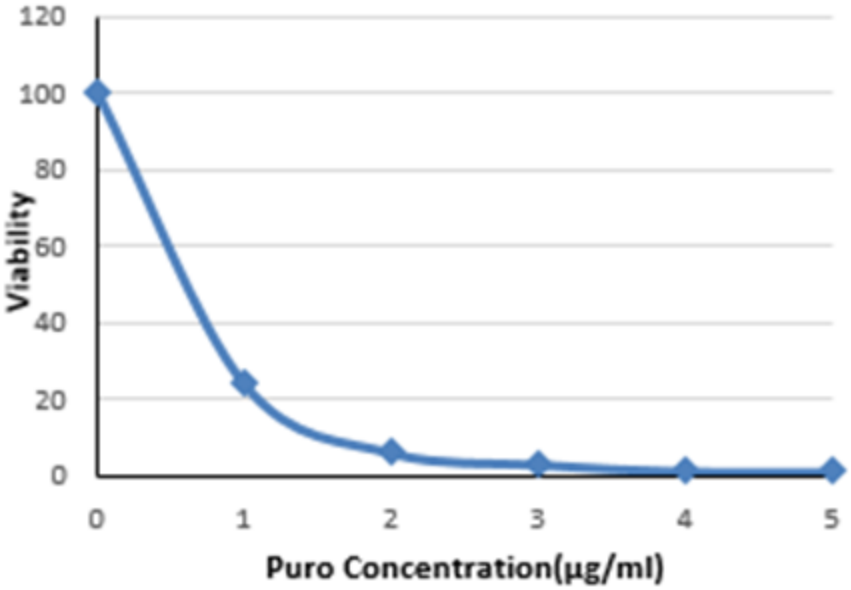


Fig 2: Example kill curve showing a 'kill concentration' of 3 µg/ml.

Guide RNA library titration of Cas9 expressing organoid lines

4m

3 **Day 1: gRNA library titration set up**

3.1 Pre-warm organoid specific culture media to room temperature and thaw a 10 mg/ml aliquot of polybrene.

3.2 Prepare transduction media, by adding 12.5 µL of [M] 10 millimolar (mM) Y-27632 (Rock inhibitor) to 50 mL organoid specific culture media ([M] 2.5 micromolar (µM) final concentration).

Note

Cells need to remain in transduction media throughout this protocol.

3.3 Collect the organoid suspension culture (from step 1.18) in a 50 ml tube and centrifuge at 800 x g for 00:02:00. 2m

3.4 Aspirate the supernatant and resuspend the pellet in 15 ml to 20 ml of TrypLE.

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

3.6 Incubate at 37 °C 5% CO_2.

3.7 Check the organoid suspension under the microscope every 15 minutes to assess and monitor the dissociation of the organoids. Mix the suspension thoroughly prior to each check to help dissociate the organoids.

Note

Generally the suspension becomes cloudy once the majority of organoids are dissociated to small clumps of cells. Stop the incubation once the organoids have broken down to single cell.

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

3.9 Aspirate the supernatant and resuspend the pellet in an appropriate volume of transduction media.

Note

15-20 ml is advised.

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

3.11 Prepare a preparation mix using the cell suspension and transduction media to achieve a final concentration of 5.6×10^6 cells in a total volume of 11.2 mL plus 14 µL 10 mg/ml polybrene.

Note

This protocol provides the volumes required to set up a 6 point titration, plus 1 well of dead volume, seeding $8x10^5$ cells/well with a final polybrene concentration of 10µg/ml. If more or less points are required adjust volumes accordingly.

3.12 Transfer 1.6 ml of the preparation mix to 6x bioreactor tubes (8×10^5 cells per tube) and add relevant transduction media volumes. (see media volume in Table 2).

Bioreactor tube	Library virus (µl) (Neat)	Media volume (µl)	Total volume added (µl)
1	0	400	400
2	5	395	400
3	10	390	400
4	20	380	400
5	60	340	400
6	120	280	400

Table 2: Library titration example volumes.

3.13 Thaw an aliquot of the gRNA library to room temperature. If the gRNA library is not being used
neat, dilute the required volume of the gRNA library in organoid complete culture media.

Note

- Any dilution made to the library at this stage will also need to be made when carrying out the gRNA library transduction at screen.
- Once defrosted, the gRNA library should be used within 1 hour.
- Avoid freeze/thaw cycles of the gRNA library.

Safety information

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

3.14 Add the appropriate volume of gRNA library virus (Table 2) to each bioreactor tube.

3.15 Mix well by pipetting and transfer the bioreactor tubes to the incubator at 37 °C 5% CO_2 for
overnight incubation.

3.16 Passage all remaining organoids in 5% BME2 organoid specific suspension culture media.

Note

The passaged organoids are to be used for gRNA library transduction set up (see section 6).

4 Day 2: Plating

4.1 Transfer the 6x bioreactor tubes to the centrifuge ensuring the centrifuge bucket lid is secure.

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 advised where possible that centrifuge buckets are sealed using safety caps and only opened in a microbiological safety cabinet.

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

4.3 Aspirate the supernatant from the tubes and resuspend each cell pellet in 1.9 mL of transduction media (prepared on Day 1) and add 100 µL of 100% BME2 to make a 5% BME2 solution. Mix well and transfer all 2 ml into each well of a low attachment 6wp. (Fig 3).

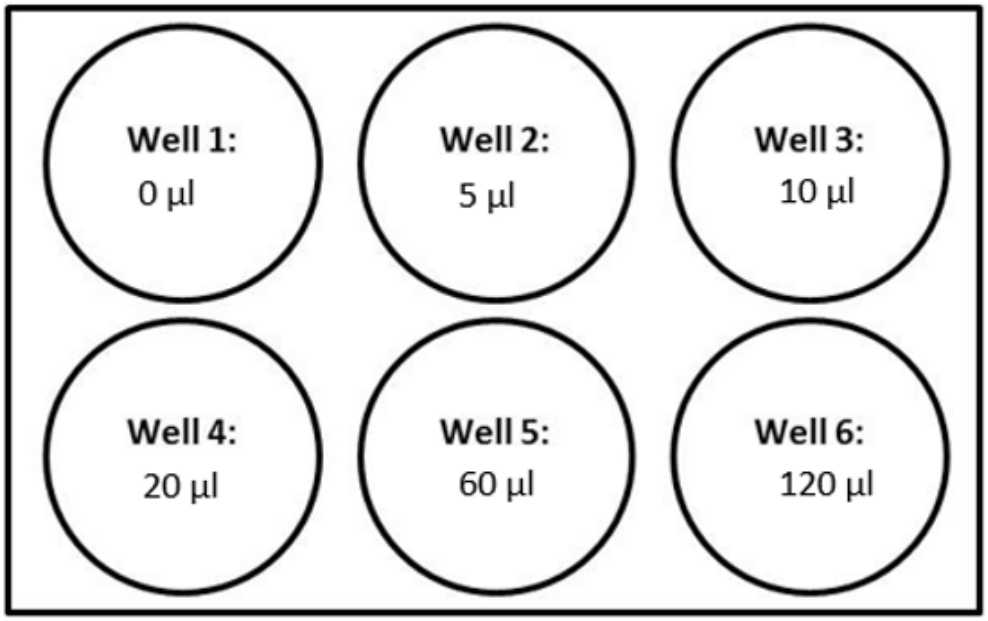


Fig 3: Example plate layout. Titration concentrations can be adjusted.

4.4 Incubate at 37 °C 5% CO_2 until Day 6.

Formaldehyde fixation of organoids

4m

5 **Day 6: Fixing and staining organoids for flow cytometry analysis.**

5.1 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).

5.2 Collect the suspension culture from each well of the ultra-low attachment 6wp into individual 2 ml tubes.

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

5.4 Aspirate the supernatant and resuspend in 1 mL of Trypsin-EDTA (0.25%).

Safety information

May cause allergy or asthma symptoms or breathing difficulties if inhaled.

5.5 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 30 minutes.

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

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

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

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

5.10 Add 1 mL PBS to each tube

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

5.12 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 a chemical fume hood, using chemical resistant gloves. Waste must be kept in the fume hood and disposed of via the recommended route.

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

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

5.15 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.

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

5.17 Using collected flow cytometry data create a plot showing side scatter area vs BFP+ expression, gating the positively expressed BFP+ cells.

5.18 For each titration point create an overlay plot (Fig 4) using your un-transduced control (Well 1 of Fig 3).

Expected result

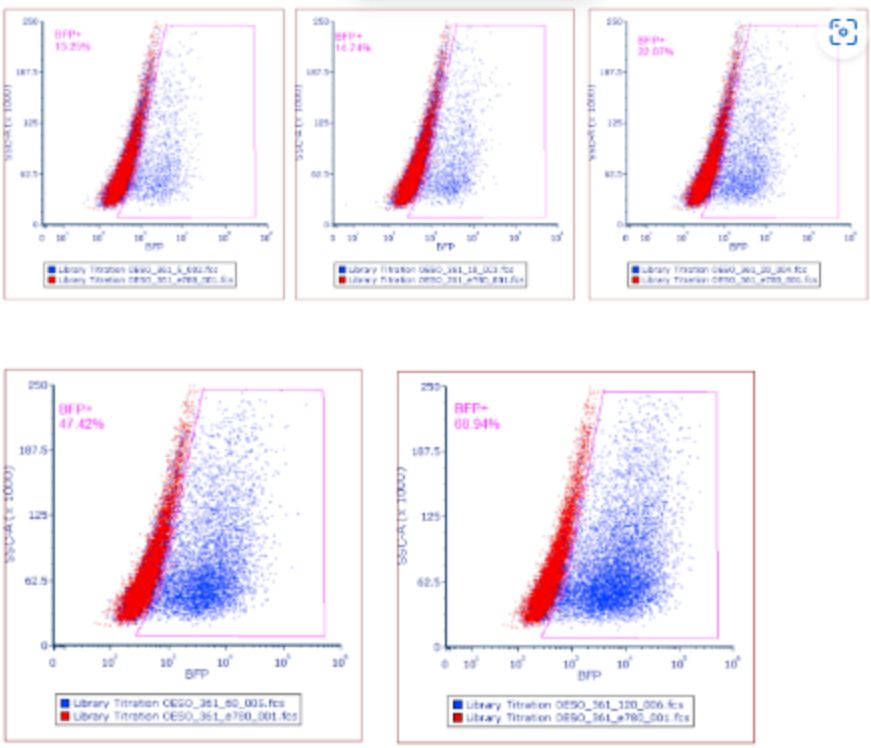


Fig 4: Example flow cytometry analysis of %BFP expression. Blue = tested virus concentration. Red = e780 un-transduced control.

5.19 Using the %BFP expression for each titration point, plot a titration curve to calculate the volume of virus required to obtain 30% BFP expression. (Fig 5).

Note

A 30% transduction efficiency is aimed for to try and ensure that each host cell has only taken up one copy of the gRNA library.

Expected result

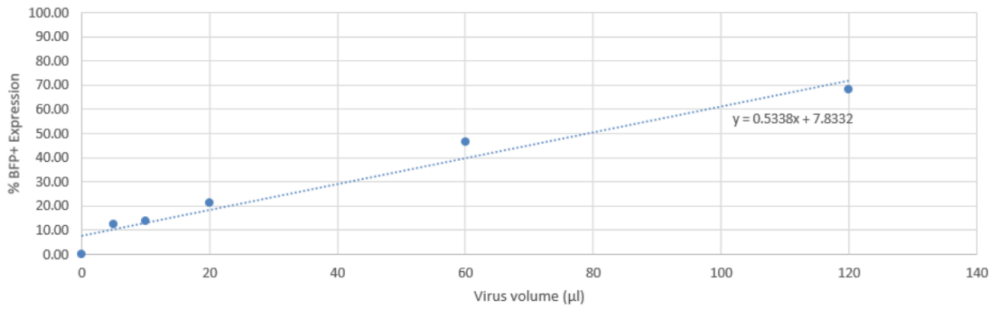


Fig 5: Example gRNA library titration curve. For 30% BFP expression a volume of 41 µl of virus per 2ml would be required.

Guide RNA Library transduction and screen of Cas9 expressing organoid lines 2m

6 **Day 1: gRNA library transduction**

6.1 Pre-warm organoid specific culture media to room temperature. Thaw a 10 mg/ml aliquot of polybrene and a [M] 10 millimolar (mM) aliquot of Y-27632 (Rock inhibitor).

6.2 Collect the organoid suspension culture from step 3.16 go to step #3.16 in a 50 ml tube and centrifuge at 800 x g for 00:02:00 . 2m

6.3 Aspirate the supernatant and resuspend the pellet in 30 ml of TrypLE.

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

6.5 Incubate at 37 °C 5% CO_2.

6.6 Check the organoid suspension under the microscope every 15 minutes to assess and monitor the dissociation of the organoids. Mix the suspension thoroughly prior to each

check to help dissociate the organoids.

Note

Generally the suspension becomes cloudy once the majority of organoids are dissociated to small clumps of cells. Stop the incubation once the organoids have broken down to single cell.

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

6.8 Aspirate the supernatant and resuspend the pellet in an appropriate volume of organoid specific culture media.

Note

25 -35 ml is advised

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

Note

- For this protocol, using the Minimal genome-wide human CRISPR Cas9 library requires a minimum of $40x10^6$ cells at this stage.
- The number of cells required may vary for transduction using alternative gRNA libraries as the number of cells required at transduction is dependent on library size and required coverage.

6.10 Thaw an aliquot of the gRNA library to room temperature. If the gRNA library is not being used
neat, dilute the required volume of the gRNA library in organoid complete culture media.

Note

- Any dilution made to the library at this stage must match the dilution carried out when setting up the gRNA library titration.
- Once defrosted, the gRNA library should be used within 1 hour.
- Avoid freeze/thaw cycles of the gRNA library.

Safety information

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

6.11 Carry out each library transduction in triplicate. Prepare each replicate in a 250 ml erlenmeyer flask as per Table 3. Mix gently by pipetting.

Note

This protocol using the Minimal genome-wide human CRISPR Cas9 library requires $12.5x10^6$ cells per replicate at transduction.

	Number of cells	Volume of polybrene (10µg/ml)	Volume of ROCKi (Y-27632) (10mM)	Volume of gRNA library virus	Volume of cell suspension	Total volume
Transduction set up per replicate	$12.5X10^6$	34 µl	8.5 µl	Library titration result scaled for transduction labware size	34 ml minus volume of gRNA library virus	34 ml

Table 3: Replicate transduction mixture set up for Minimal genome-wide human CRISPR Cas9 library. The volume of library virus required to obtain 30% transduction efficiency will be previously established following library titration and scaled to account for transduction labware size at screen.

6.12 Prepare an un-transduced control using the appropriate volumes of reagents listed in Table 4. Approximately 1.6 $\times 10^6$ cells should be seeded into a 50 ml bioreactor tube.

Number of cells	Volume of polybrene (10µg/ml)	Volume of ROCKi (Y-27632) (10mM)	Volume of cell suspension	Total Volume
$1.6X10^6$	4 µl	1 µl	4 ml	4 ml

Table 4: Un-transduced control mixture set up.

6.13 Incubate the control bioreactor and replicate flasks prepared in step 6.11 and 6.12 overnight at 37 °C 5% CO_2.

7 Day 2: Plating

7.1 2m

Collect and transfer the replicate solutions into labelled 50 ml tubes and centrifuge alongside the control bioreactor tube at 800 x g for 00:02:00 .

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 advised where possible that centrifuge buckets are sealed using safety caps and only opened in a microbiological safety cabinet.

7.2 Aspirate the supernatant and resuspend each replicate pellet in 19 mL organoid specific culture media with 5 µL of [M] 10 millimolar (mM) Y-27632 (Rock inhibitor) and 1 mL of BME2 to make a 5% BME2 solution.

7.3 Mix the suspension well by gentle pipetting and transfer each replicate into x1 ultra low-attachment T75 flask and incubate at 37 °C 5% CO_2.

7.4 For the control sample, aspirate the supernatant from the tube and resuspend the cell pellet in 1.9 ml of organoid specific culture media containing 0.5 µL

[M] 10 millimolar (mM) Y-27632 (Rock inhibitor) and add 100 µL of BME2 to make a 5% BME2 solution.

7.5 Mix well by pipetting and transfer into 1 well of an ultra-low attachment 6wp and incubate at 37 °C 5% CO_2.

8 **Day 6: Puromycin selection and flow cytometry analysis.**

8.1 Using a pipette, collect 0.5-1 ml from each replicate and transfer to individual 2 ml tubes. Collect all of the control and transfer to another 2 ml tube, this is done to test infection efficiency using flow cytometry.

8.2 Fix each organoid sample and analyse by flow cytometry following steps 5.1 to 5.17. 5.1

Note

The un-transduced control once fixed should be kept at 4 °C for re-analysis at Day 21-23 (Assessment of final selection efficiency).

8.3 The pass rate for transduction efficiency is 30%, calculated from step 5.19 go to step #5.19 . However a value between 15-50% is acceptable. This is to try and ensure only 1 copy of the library has been transduced per cell. If a value outside of this range is obtained at this stage the screen should be failed. (Fig 6). If the pass rate has been reached, continue to step 8.4.

Expected result

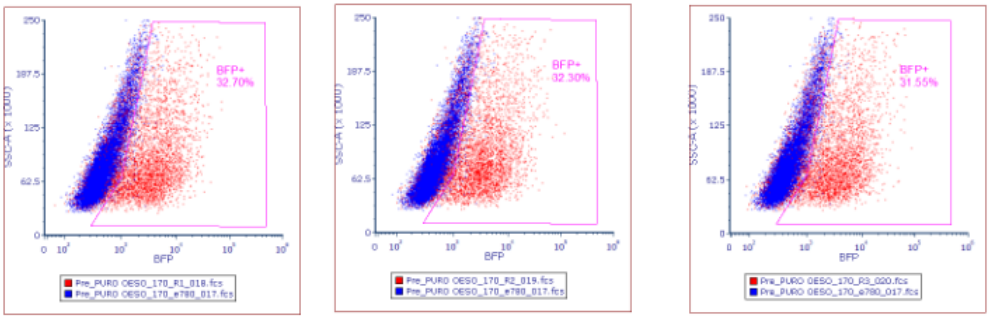


Fig 6: Example flow cytometry analysis of transduction efficiency for screen replicates showing %BFP expression. Red = transduced replicate. Blue = e780 un-transduced control.

8.4 For each replicate; collect the suspension culture in a 50 ml tube using a stripette or by pouring the suspension.

Note

If pouring the suspension into a 50 ml tube be mindful of the increased contamination risk.

8.5 Mix the suspension well by pipetting to break down any larger clumps of BME2/aggregates
before centrifugation.

8.6 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 advised where possible that centrifuge buckets are sealed using safety caps and only opened in a microbiological safety cabinet.

8.7 Aspirate the supernatant and resuspend the pellet in 10 mL organoid specific culture media.

8.8 Prepare the ultra-low attachment T75 flask with the appropriate remaining volume of media, BME2 and puromycin (Table 5 and 6) (Puromycin should be added at a concentration previously calculated in go to step #2.3 Fig 2).

Note

Ultra-low attachment T75 flasks have a working volume of between 20 - 40 ml. This is dependent on the confluency of the organoid suspension culture.

Safety information

Puromycin is toxic if swallowed, harmful in contact with skin

Total volume per flask (ml)	Cell suspension (ml)	Volume of media added to flask (ml)	Volume of BME2 (ml)
20	10	9	1
30	10	18.5	1.5
40	10	28	2

Table 5: Volumes for preparing 5% BME media solution

Final puromycin concentration (µg/ml)	Flask volume 20 ml	Flask volume 30 ml	Flask volume 40 ml
1	20 µl	30 µl	40 µl
2	40 µl	60 µl	80 µl
3	60 µl	90 µl	120 µl
4	80 µl	120 µl	160 µl
5	100 µl	150 µl	200 µl

Table 6: Puromycin selection volumes to add per specific total flask volumes.

8.9 Mix the cell suspension well by pipetting, then transfer cell suspension to the individual ultra-low attachment T75 flasks.

8.10 Incubate at 37 °C 5% CO_2.

9 **Day 9-20: Maintenance of screens. (The following steps outline a 1:2 passage)**

Note

Media change, or passage 1:2 during week 3 as appropriate.
Do not discard any cells at this point and re-seed all at passage. Screens are maintained in puromycin selection until the point of pellet.

9.1 Collect suspension for each replicate in 50 ml tubes.

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

Safety information

Centrifuge buckets must be sealed using safety caps, which must only be opened in a microbiological safety cabinet.

9.3 Aspirate the supernatant and resuspend the pellet in an appropriate volume of TrypLE.

Note

Small pellets (less than 2 ml in size) can be resuspended in up to 10 ml, whilst larger pellets may need to be resuspended in up to a maximum of 40 ml per tube.

9.4 Mix well by pipetting and incubate at 37 °C 5% CO_2 for 00:10:00 . 10m

Note

Check organoid suspension under the microscope after 5 minutes to assess and monitor the dissociation of the organoids. Pipette the cell suspension up and down to help dissociate the organoids. Generally the suspension becomes cloudy once the majority of organoids are dissociated to smaller clumps of cells.

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

Safety information

Centrifuge buckets must be sealed using safety caps, which must only be opened in a microbiological safety cabinet.

9.6 Aspirate the supernatant and resuspend each pellet in 20 mL organoid specific culture media.

9.7 Prepare 2x ultra-low attachment T75 flasks with the appropriate remaining volume of media, BME2 and puromycin per replicate (Table 5 and 6). 8.8

9.8 Mix the cell suspension well by pipetting, then transfer half the cell suspension to each new ultra-low attachment T75 flask.

9.9 Incubate at 37 °C 5% CO_2.

10 **Day 21-23: Assessment of final selection efficiency**

10.1 Using a pipette, collect 0.5-1 ml from each replicate and transfer to separate 2 ml tubes.

10.2 Fix each organoid sample and analyse by flow cytometry following steps 5.1 to 5.17. 5.1

Note

The un-transduced control, fixed at Day 6 go to step #8.2 and kept at 4 °C should be re-analysed alongside the fixed replicates at Day 21-23.

10.3 The pass rate for post-puromycin selection is between 60-100%. This ensures efficient selection of organoids that have been transduced with the viral library. If a value below

this range is obtained at this stage the screen should be failed. (Fig 7). If the pass rate has been reached continue to step 11.1.

Expected result

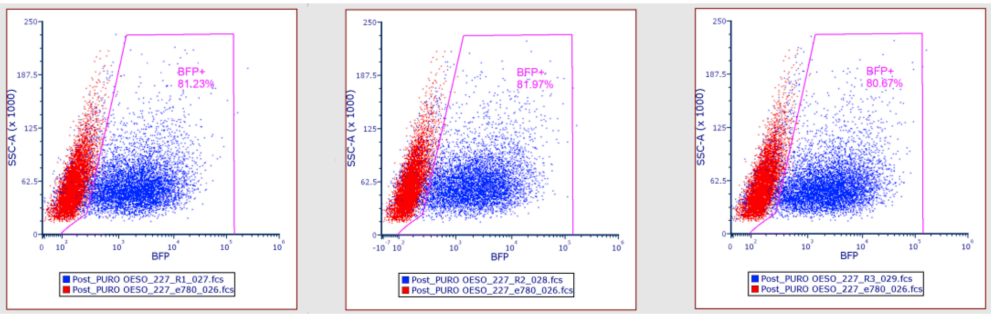


Fig 7: Example flow cytometry analysis of screen replicates at post-puromycin selection showing %BFP expression. Blue = transduced replicate. Red = e780 un-transduced control.

11 Day 21-23: Pelleting using Cell Recovery Solution

11.1 Collect suspension for each replicate in 50 ml tubes.

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

Safety information

Centrifuge buckets must be sealed using safety caps, which must only be opened in a microbiological safety cabinet.

11.3 Aspirate the supernatant and resuspend each pellet in an appropriate amount of Cell Recovery Solution (up to 30 mL per replicate) to remove BME. Mix well by pipetting.

11.4 Incubate on ice for 01:00:00 . 1h

11.5 Once the sample tubes have been on ice for 00:30:00 ; mix the suspensions well by pipetting and take aliquots from each replicate to perform a cell count. 30m

11.6 Following 01:00:00 on ice, based on the required number of cells per pellet, transfer the required volume of cells per pellet into 15 ml tubes. 1h

Note

For the minimal library used in this example $25x10^6$ cells are recommended per pellet. If more than 15 ml of suspension is required, multiple rounds of centrifugation are advised.

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

11.8 Aspirate the supernatant and resuspend in 10 ml of 4 °C PBS to wash and remove the Cell Recovery Solution from the pellets.

Note

It is advised to dispense the PBS quickly onto the pellet and to avoid mixing the suspension as the pellet will be prone to sticking to the stripette resulting in a loss of cells.

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

11.10 Aspirate the PBS and store the pellets at -80 °C ready for downstream processing.

Protocol references

Verity Goodwin, Emily Souster, Charlotte Beaver, Adam Jackson, Rizwan Ansari, Mathew Garnett , Fiona Behan 2020. Guide RNA Library Transduction of Cas9 Cancer Cell Lines. **protocols.io** dx.doi.org/10.17504/protocols.io.bg2njyde

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