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Assessment of Cas9 Activity in Cas9 Transduced Cancer Cell Lines

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1 Works for me dx.doi.org/10.17504/protocols.io.bgvtjw6n

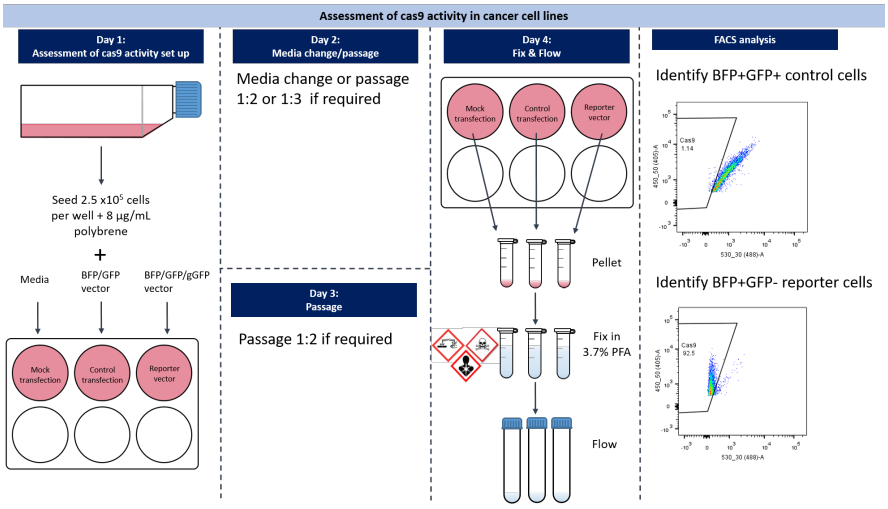
Cellular Generation and Phenotyping

Verity Goodwin

ABSTRACT

Cas9 expressing cancer cell lines need to be assessed for cas9 activity to ensure they are capable of efficiently knocking out genes. The activity is assessed by transducing the cells with a BFP-GFP vector containing an anti-GFP guide RNA. If the cas9 is active, the GFP will be knocked down so these cells will express BFP but not GFP as detected by flow cytometry.

Process diagram:



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GUIDELINES

Cas9 cancer cell lines should be produced following the protocol: Cas9 Transduction of Cancer Cell Lines. Cas9 lines should be over 50% confluent in a T150 flask within 4 weeks of transduction to be assessed for cas9 activity.

This protocol takes 4 days.

$1x10^6$ cells are required for this protocol.

MATERIALS

NAME	CATALOG #	VENDOR
DPBS	14190	Invitrogen - Thermo Fisher
TrypLE™ Express Enzyme (1X), no phenol red	12604021	Thermo Fisher
6 Well Clear TC-Treated Multiple Well Plates	3516	Corning
Polybrene Infection / Transfection Reagent	TR-1003-G	Emd Millipore
Falcon Round bottomed 5ml tube with cell strainer lid	734-0001	VWR international Ltd
Microcentrifuge tube Safe-Lock write-on 1.5mL Eppendorf Tube	0030 120.086	Eppendorf

MATERIALS TEXT

Select an appropriate culture media for your cell line. Common culture medias used for cancer cell lines are serum supplemented Advanced DMEM D-12 or RPMI in the presence of pen-strep.

BFP/GFP/gGFP Vector (Reporter Vector): lentiviral reporter pKLV2-U6gRNA(gGFP)-PGKBFP2AGFP-W
BFP/GFP Vector (Control Vector): pKLV2-U6gRNA(Empty)-PGKBFP2AGFP-W
- Further information regarding these vectors can be found in Figure S1:
Tzelepis K, Koike-Yusa H, De Braekeleer E, et al. A CRISPR Dropout Screen Identifies Genetic Vulnerabilities and Therapeutic Targets in Acute Myeloid Leukemia. *Cell Rep.* 2016;17(4):1193-1205. doi:10.1016/j.celrep.2016.09.079

Equipment

- Microbiological Safety Cabinet (MSC)
- Centrifuge
- Microfuge
- Pipetboy
- Stripettes
- P1000 pipette and tips
- **37 °C** waterbath
- **37 °C** humidified incubator (5% CO2)
- Light microscope

SAFETY WARNINGS

Chemical safety warnings:

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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.	
Blasticidin	Toxic if swallowed, harmful in contact with skin	

Biological safety warnings:

- Cell lines may contain adventitious agents, including viruses. No attempt will be made to culture these agents deliberately. Correct use of PPE will drastically reduce the risks.
- Lentiviruses used in this protocol can infect human cells but are non-replicating and therefore the pathogenicity of these viruses is negligible. Correst use of PPE will drastically reduce the risks.

BEFORE STARTING

Pre-warm cell culture media to room temperature
Thaw 10mg/mL polybrene to room temperature
Thaw BFP/GFP Vector (Control Vector) to room temperature
Thaw BFP/GFP/gGFP Vector (Reporter Vector) to room temperature

Day 1

1 Detach and collect cas9 cells as per protocol "Passaging adherent cancer cell lines" steps 1-8 found here: https://protocols.io/view/passaging-adherent-cancer-cell-lines-bgtbjwin.html

2 Prepare a cell suspension containing 1x10^6 cells in **7.2 mL** media (this equates to 2.5x10^5 cells per well in **1.8 mL**).

2.1 Add **6.4 µl** polybrene (8µg/ml concentration) to the cell suspension and mix using a 10ml stripette and pipetboy.

3 Transfer **1.8 mL** of the cell suspension into 3 wells of a 6 well plate to include a mock, control, and reporter transduction with the final cell number and reagent volumes shown in table 1. Ensure the plate is clearly labelled as per Figure 1.

Well	Transfection	Number of Cells	Cell + polybrene suspension (ml)	Vector
1	Mock	2.5 x10^5	1.8ml	200µl media
2	Control	2.5 x10^5	1.8ml	200µl BFP/GFP
3	Reporter	2.5 x10^5	1.8ml	200µl BFP/GFP/gGFP

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Table 1. Reagents per well of a 6 well plate for transduction set up.

image001.png

Figure 1. 6 well plate layout

4 Add **200 µl** of media to the mock well, and **200 µl** of the designated control or reporter virus to the appropriate well (see table 1). Mix well by rocking the plate gently.

5 Place the plate in the incubator.

Day 2

6 if cells are <90% confluent, carefully replace media with **2 mL** fresh media per well, without dislodging the cells.

7 If cells are >90% confluent, passage each well:

7.1 Aspirate culture media from each well, and carefully wash each well with **2 mL** PBS by angling the pipette at the side of the wells. Aspirate PBS.

7.2 Add **500 µl** TrypLE to each well and place in the incubator for 3-5 minutes. Use the microscope if necessary to check that all the adherent cells have been dislodged. If not, return them to the incubator for a few more minutes until detached.

7.3 When cells have detached from the base of the well, add **500 µl** complete media to each well. Mix by pietting up and down using a P1000, and wash the base of the well ensuring cells are fully detached.

7.4 Collect the 1ml cell suspension in a labelled 1.5ml centrifuge tube and centrifuge **300 x g 00:03:00**

Centrifuge using a minifuge inside the MSC.

7.5 Carefully aspirate the supernatent without disturbing the cell pellet. Resuspend each pellet in an appropriate volume of media to carry out either a 1:2 or 1:3 split ratio, dependent on growth rate. Discard extra cells.

Day 3

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8 If cells are >90% confluent, passage at a 1:2 split ratio, following steps 7.1 - 7.5.

Day 4: Fixing

9 Detach and collect cells following steps 7.1 - 7.4. Carefully aspirate supernatent without disturbing the pellet.

10 Follow "Fixing cell pellets" protocol found here: https://protocols.io/view/fixing-cell-pellets-bg2fjybn.html

Flow Cytometry

11 Set up an experiment ensuring that parameters FSC-A, FSC-W, SSC-A, 450/50-A (BFP) and 530/30-A (GFP) are selected.

12 Run the mock sample first and use this to separate debris (FSC-A vs SSC-A) and identify single cells (FSC-A vs FSC-W). Use the polygon gate to select for this cells (see Figure 2). If required, adjust FSC and SSC voltages to ensure the main population lies within the plot.

figure 1.PNG

Figure 2. Setting up plots and gates for the mock sample.

12.1 Check that the mock cells are negative for BFP and GFP using the filters: BFP 450/50 (405)-A and GFP 530/30 (488)-A and insert a polygon gate with the negative population positioned on the left side (Figure 2, bottom left panel). Run and record 10,000 events per sample.

13 Next, run the control sample. The control sample should contain cells that are positive for both BFP and GFP. Run and record 10,000 events.

14 Finally, run and record 10,000 events for the reporter sample.

15 Export the .fcs files. Carry out analysis using appropriate analysis software.

FACS Analysis

16 Using the analysis software, start by analysing the mock sample. Firstly, identify single cells by drawing a gate around the main population in FSC-A vs SSC-A, as shown in Figure 2, top left panel.

The following steps are for the use of FlowJo analysis software but can be adapted for use on other analysis software.

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16.1 Double-click inside the gate to open a new pane containing the selected cells. Identify single cells by drawing a gate around the population shown in Figure 2, top right panel.

16.2 Double-click inside the gate to open a new pane containing single cells.

16.3 Change the plot axis to show BFP vs SSC-A and draw a BFP+ gate based on the negative cells, see Figure 3, left panel.

Cas9 activity is defined as the percentage of cells that have taken up the vector that have silenced GFP expression, so we need to identify BFP positive cells that are GFP negative.

figure 2.PNG

Figure 3. Setting BFP+ gates for the mock and control cells

17 Copy these gates across to the control sample and check that it identifies BFP+ cells as shown in Figure 3, right panel.

17.1 Using the control cells, double-click on the BFP+ gate population and change the plot axis to BFP vs GFP as shown in Figure 4, left panel. Gate the region of BFP+ cells that are not GFP+. Name this gate Cas9.

figure 3.PNG

Figure 4. Identifying Cas9 positive cells

18 Copy all the gates over to the reporter sample. Check that the Cas9 gate does not include any GFP+ cells. The majority of cells should have shifted to BFP+ GFP-. This will provide the percentage of cas9 positive cells in the cas9 cell line.

Pass criteria: 75% cas9 positivity.

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