

Jul 24, 2020

## Assessment of Cas9 Activity in Cas9 Transduced Cancer Cell Lines

DOI

[dx.doi.org/10.17504/protocols.io.bgvtyw6n](https://dx.doi.org/10.17504/protocols.io.bgvtyw6n)

Verity Goodwin<sup>1</sup>, Emily Souster<sup>1</sup>, Charlotte Beaver<sup>1</sup>, Adam Jackson<sup>1</sup>, Rizwan Ansari<sup>1</sup>, Mathew Garnett<sup>1</sup>, Fiona Behan<sup>1</sup>

<sup>1</sup>Wellcome Sanger Institute

Cellular Generation and ...



Verity Goodwin

OPEN  ACCESS



DOI: [dx.doi.org/10.17504/protocols.io.bgvtyw6n](https://dx.doi.org/10.17504/protocols.io.bgvtyw6n)

**Protocol Citation:** Verity Goodwin, Emily Souster, Charlotte Beaver, Adam Jackson, Rizwan Ansari, Mathew Garnett, Fiona Behan 2020. Assessment of Cas9 Activity in Cas9 Transduced Cancer Cell Lines. **protocols.io**

<https://dx.doi.org/10.17504/protocols.io.bgvtyw6n>

**License:** This is an open access protocol distributed under the terms of the **[Creative Commons Attribution License](#)**, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

**Protocol status:** Working

**We use this protocol and it's working**

**Created:** May 27, 2020

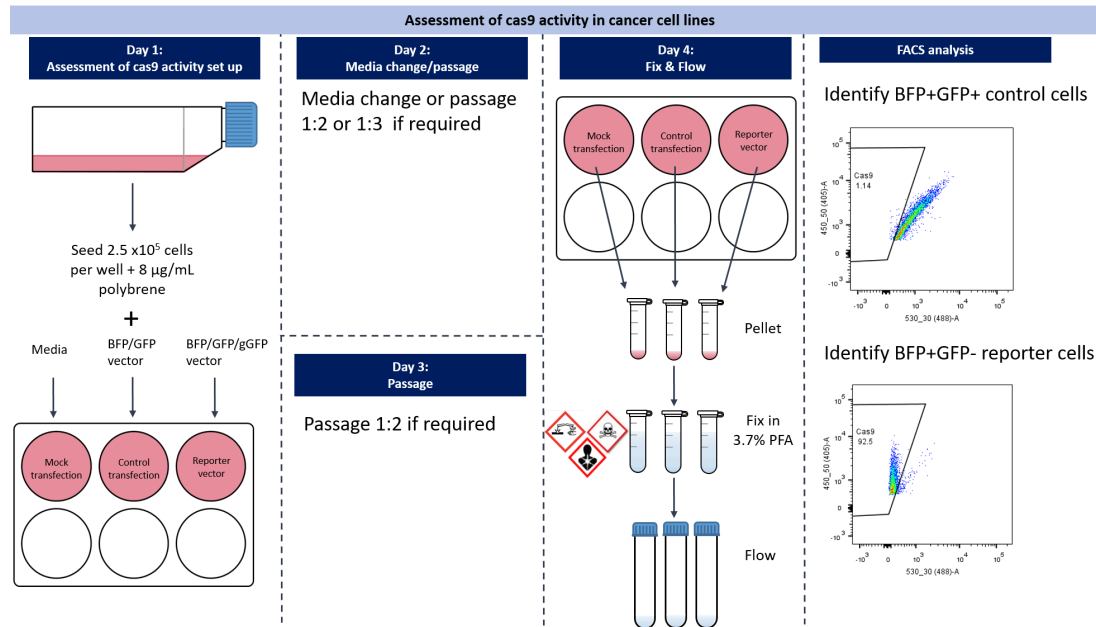
**Last Modified:** July 24, 2020

**Protocol Integer ID:** 37523

## 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:



## 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.

$1 \times 10^6$  cells are required for this protocol.



## Materials

### MATERIALS

✕ DPBS Invitrogen - Thermo Fisher Catalog #14190

✕ TrypLE<sup>®</sup>; Express Enzyme (1X), no phenol red Thermo Fisher Catalog #12604021

✕ 6 Well Clear TC-Treated Multiple Well Plates Corning Catalog #3516

✕ Polybrene Infection / Transfection Reagent Emd Millipore Catalog #TR-1003-G

✕ Falcon Round bottomed 5ml tube with cell strainer lid VWR international Ltd Catalog #734-0001

✕ Microcentrifuge tube Safe-Lock write-on 1.5mL Eppendorf Tube Eppendorf Catalog # 0030 120.086

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% CO<sub>2</sub>)
- Light microscope

## Safety warnings

### ! Chemical 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.	
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 start





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  $1 \times 10^6$  cells in  7.2 mL media (this equates to  $2.5 \times 10^5$  cells per well in  1.8 mL ).
- 2.1 Add  6.4  $\mu$ L polybrene (8 $\mu$ g/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	$2.5 \times 10^5$	1.8ml	200 $\mu$ l media
2	Control	$2.5 \times 10^5$	1.8ml	200 $\mu$ l BFP/GFP
3	Reporter	$2.5 \times 10^5$	1.8ml	200 $\mu$ l BFP/GFP/gGFP

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

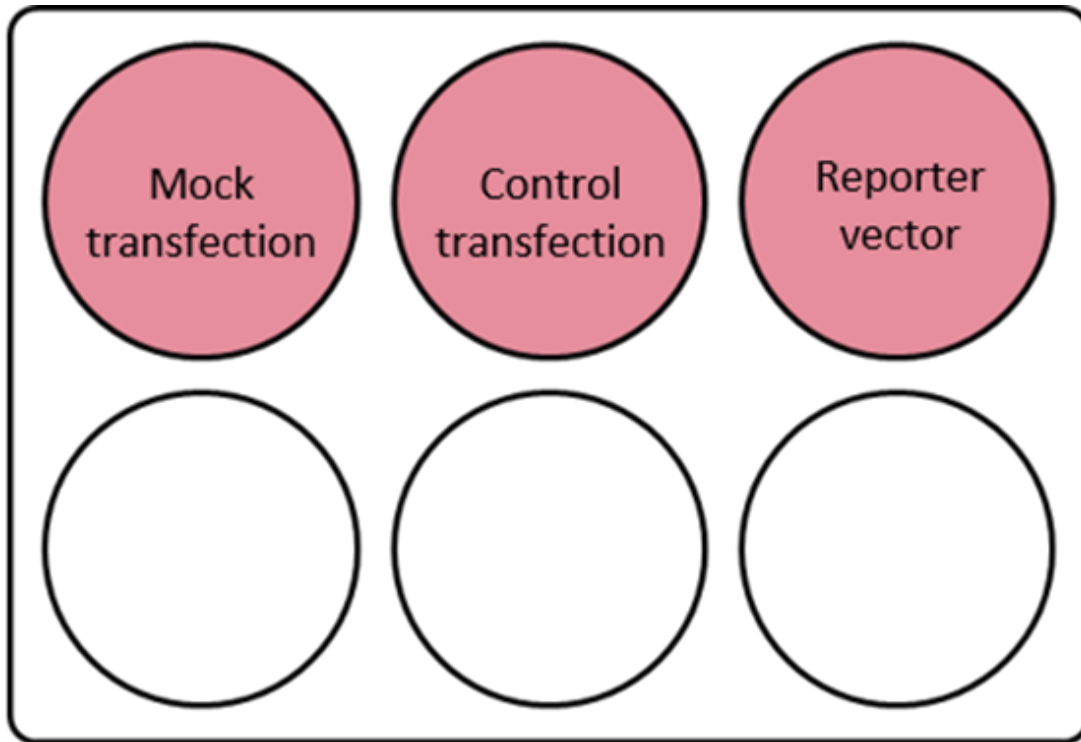







Figure 1. 6 well plate layout


- 4 Add  200  $\mu\text{L}$  of media to the mock well, and  200  $\mu\text{L}$  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  $\mu$ L 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  $\mu$ L complete media to each well. Mix by pipetting 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

#### Note

Centrifuge using a minifuge inside the MSC.

7.5 Carefully aspirate the supernatant 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

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 supernatant 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.

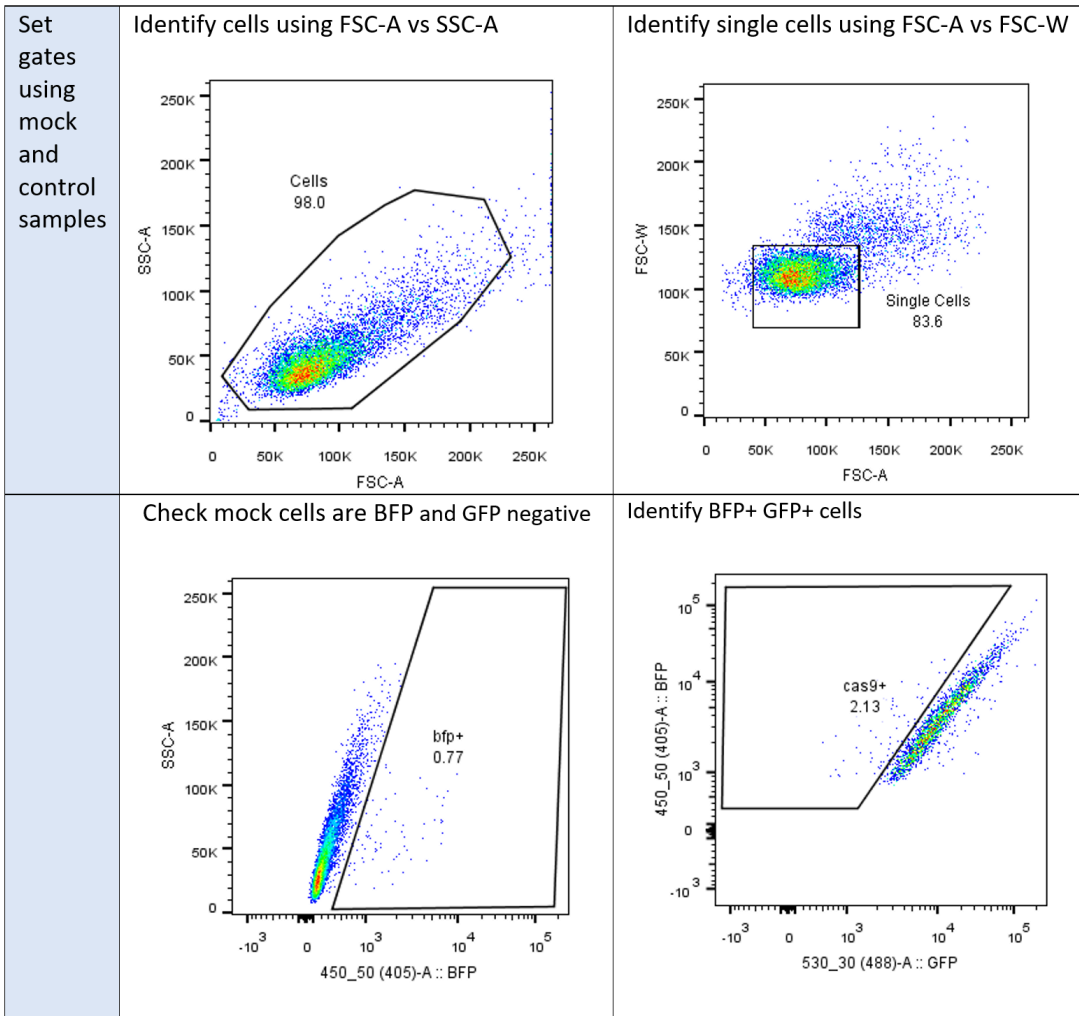


Figure 2. Setting up plots and gates for the mock sample.

- 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.

### Note

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

- 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.

### Note

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.

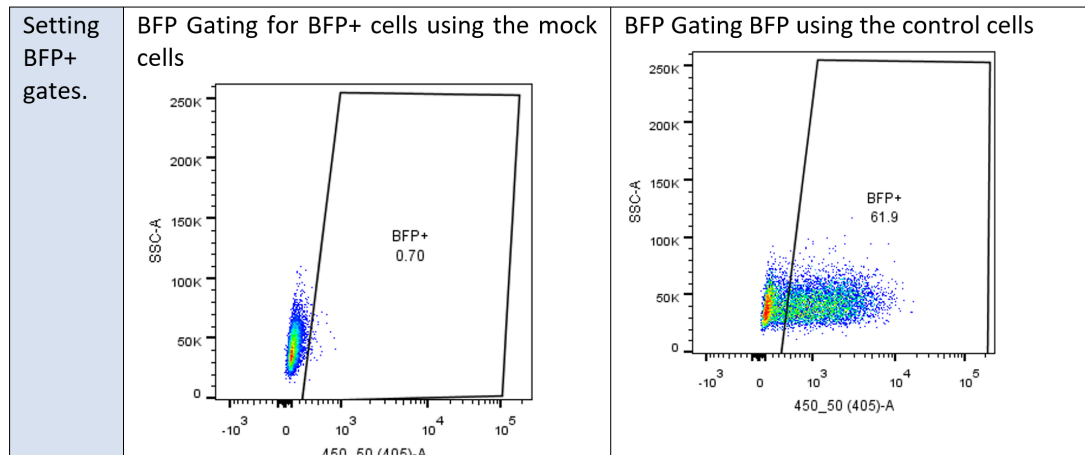


Figure 3. Setting BFP+ gates for the mock and control cells

- 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.

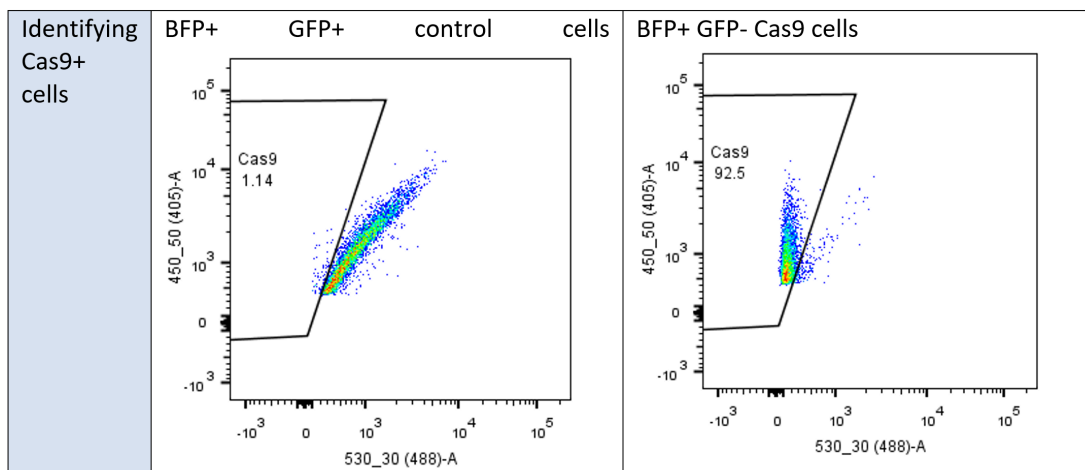


Figure 4. Identifying Cas9 positive cells

- 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.



#### Note

Pass criteria: 75% cas9 positivity.