Jul 23, 2020

Guide RNA Library Titration of Cas9 Cell Lines

DOI
dx.doi.org/10.17504/protocols.io.bgxujxnw

Verity Goodwin[1], Emily Souster[1], Charlotte Beaver[1], Adam Jackson[1], Rizwan Ansari[1], Mathew Garnett[1], Fiona Behan[1]

[1]Wellcome Sanger Institute

Cellular Generation and ...

Verity Goodwin

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Protocol Citation: Verity Goodwin, Emily Souster, Charlotte Beaver, Adam Jackson, Rizwan Ansari, Mathew Garnett, Fiona Behan 2020. Guide RNA Library Titration of Cas9 Cell Lines. **protocols.io https://dx.doi.org/10.17504/protocols.io.bgxujxnw**

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

Created: May 29, 2020

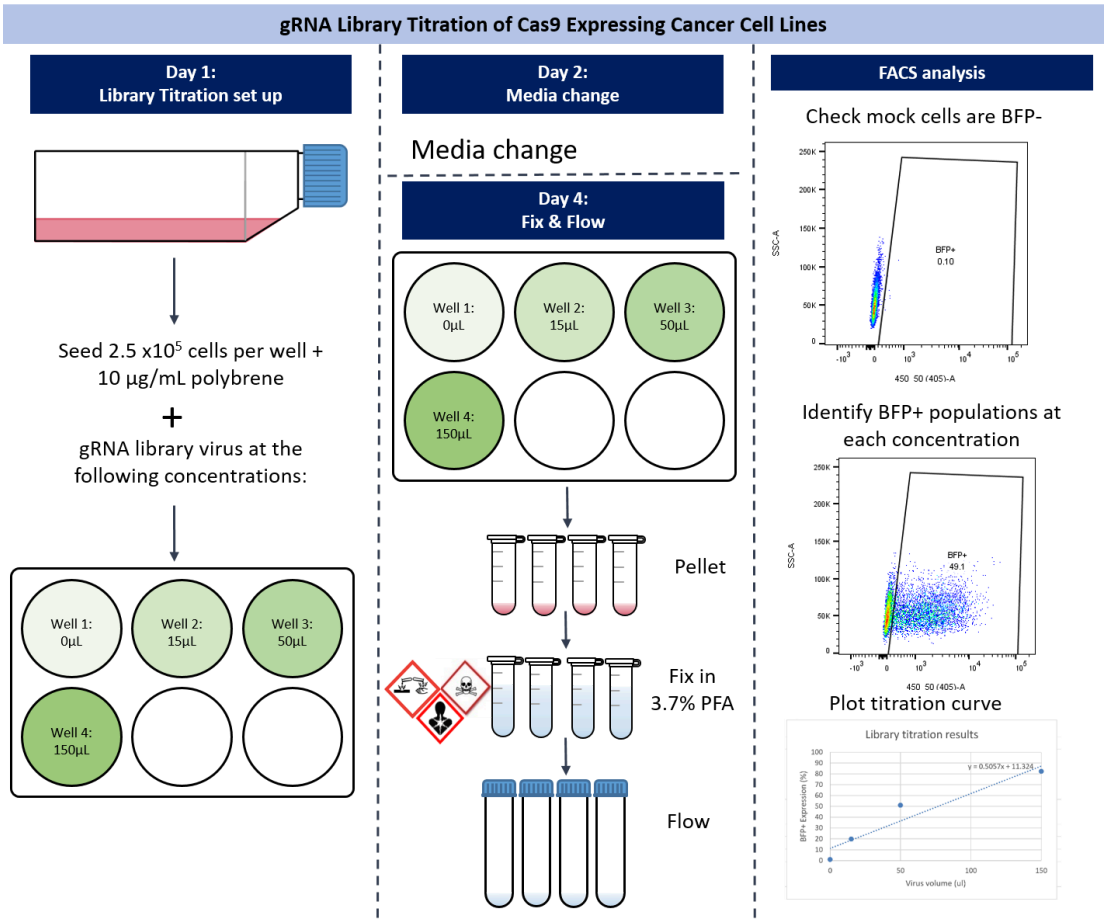
Last Modified: July 23, 2020

Protocol Integer ID: 37588

Abstract

This protocol is for the gRNA library titration of cas9 expressing cancer cell lines using the Kusuke Yusa v1.1 whole genome gRNA library. gRNA library titration is performed on all the cas9 cancer cell lines prior to gRNA library screening. The library titration allows us to determine the volume of library virus required to transduce cas9 cancer cells at 30% transduction efficiency which is calculated using FACS analysis.

Process diagram:



Guidelines

This protocol is for the titration of the 'commercially available' Kusuke Yusa v1.1 whole genome gRNA library. It can be adapted for other gRNA libraries, under the assumption that there is a BFP reporter in the gRNA library. The aim of this protocol is to identify how much virus to dose each cell line for transduction to avoid host cells taking up more than one gRNA, therefore an MOI of 30% should be aimed for.

The volumes of library titrated may differ depending on the batch of virus, it may require diluting prior to titrating. It may also differ for different libraries. An increased number of titration points may also be required. This may require some R&D to determine optimal volumes to titrate.

This protocol takes 4 days.

1.25×10^6 cells are required for this protocol.

Materials

MATERIALS

DPBS **Invitrogen - Thermo Fisher Catalog #**14190

TrypLE™ 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

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

Human Improved Genome-wide Knockout CRISPR Library **addgene Catalog #**67989

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.

Equipment

- Microbiological Safety Cabinet (MSC)
- Centrifuge
- Microfuge
- Media dispensing pump (recommended)
- Pipetboy
- Stripettes
- P1000 pipette and tips
- P200 pipette and tips
- P20 pipette and tips
- 37 °C waterbath
- 37 °C humidified incubator (5% CO2)
- Light microscope

Safety warnings

Chemical safety warnings:

Chemical	Hazards	Hazard pictogram
Virkon	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.	

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 culture media to room temperature
- Thaw polybrene (10mg/ml)
- Thaw virus (the virus should only be thawed twice)
- All lentiviral waste should be deactivated with 1% Virkon solution for a minimum of 1 hour.

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 1.25×10^6 cells in 8 mL complete media to make a final concentration of 1.56×10^6 cells per ml.

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

3 Transfer 1.6 mL of the cell suspension into 4 wells of a 6 well plate (this equates to 2.5×10^5 cells per well) and add the relevant media volume, see table 1, column 4.

	Titration Volume (µl)	Cell + polybrene suspension (ml)	Volume of media (ml)
	0	1.6	400
	15	1.6	385
	50	1.6	350
	150	1.6	250

Table 1. Library titration volumes, per well of a 6 well plate

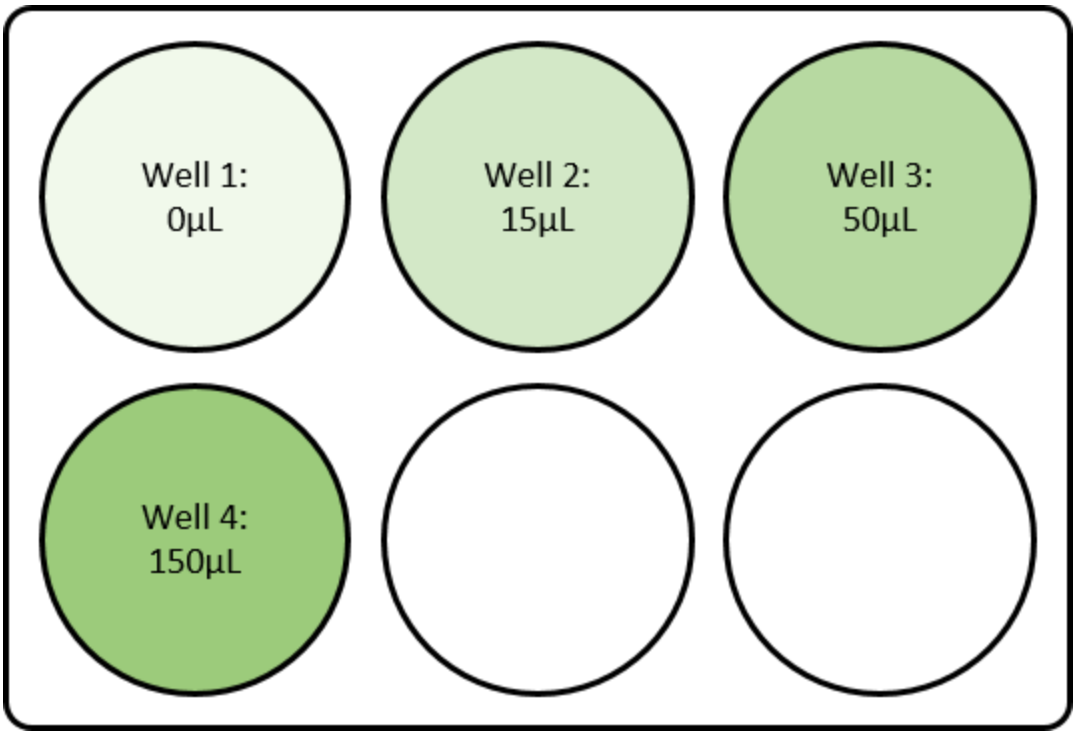


FIgure 1. Library titration plate layout

4 Dilute the gRNA library in complete media if required. **The defrosted gRNA library should be stored on ice and used within 1 hour.**

Safety information

Lentivirus: 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.

5 Add the diluted gRNA library volumes in Table 1 to the 6 well plate according to the layout in Figure 1. Mix well by rocking the plate gently.

6 Place the plate in the incubator.

Day 2

7 Carefully replace media with 2 mL fresh media per well, without dislodging the cells.

Day 4: Fixing

8 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.

> Note
>
> Day of harvest may be adjusted if cell lines take longer to express BFP. Some optimisation may be required.

9 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.

10 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.

11 Collect the 1ml cell suspension in a labelled 1.5ml centrifuge tube and centrifuge 300 x g, 00:03:00. Carefully aspirate the supernatent without dislodging the pellet.

> Note
>
> Centrifuge using a minifuge inside the MSC.

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

Flow Cytometry and analysis

13 Set up an experiment ensuring parameters: FSC-A, FSC-W, SSC-A, 450/50-A (BFP) are selected.

Note

The following steps are for use on the BD LSRFortessa flow cytometer. It can be adapted for use on other flow cytometers.

14 Run the mock sample (0) 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 these cells (see Figure 2, top panel). If required, adjust FSC and SSC voltages so that the main population lies within the plot.

Note

FSC will need to be reduced for very large cell lines.

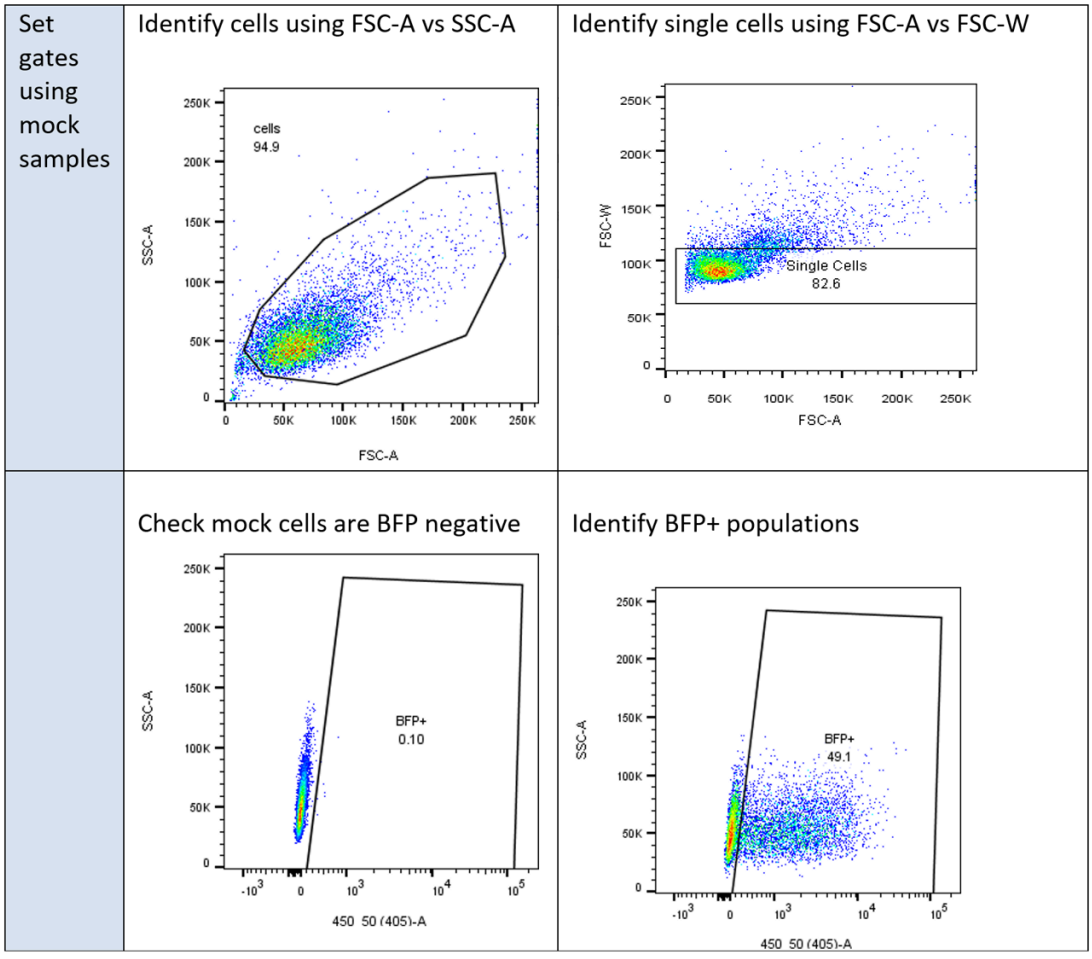


Figure 2. Setting up plots and gates for the mock sample

15 Check that the mock cells are negative for BFP using 450/50 (405)-A vs SSC-A and insert a polygon gate with the negative population positioned to the left of the gate, see Figure 2, bottom left panel. Run and record 10,000 events per sample.

16 Next run the titrated samples, starting with the lowest volume of virus (15µl). The BFP positive cells should appear in the BFP+ gate, shown in Figure 2, bottom right panel. Run and record 10,000 events per sample.

Note

Library infectivity is defined as the percentage of single cells that are BFP positive.

17 Export FCS files. Carry out analysis using appropriate analysis software, such as FlowJo. Analysis and gating protocol are the same used to set up the flow cytometer as shown in Figure 2.

18 Export data as the percentage of single cells that are BFP positive as an excel file for each titration.

19 Plot a titration curve in Microsoft Excel by plotting the percentage of BFP% cells at each virus concentration, to calculate the amount of virus required for 35% transduction efficiency based on the linear portion of the curve. See Figure 3.

Note

Although the transduction efficiency of 30% is ideal, we aim for 35% when scaling up to account for errors when increasing the surface area from a 6 well plate to a flask.
For example, in Figure 3, the equation to determine the amount of virus that infects 35% of cells in one well of a 6 well plate is (35-11.324)/0.5057= 48.8µl. This value can then be scaled up to the desired surface area for transduction.

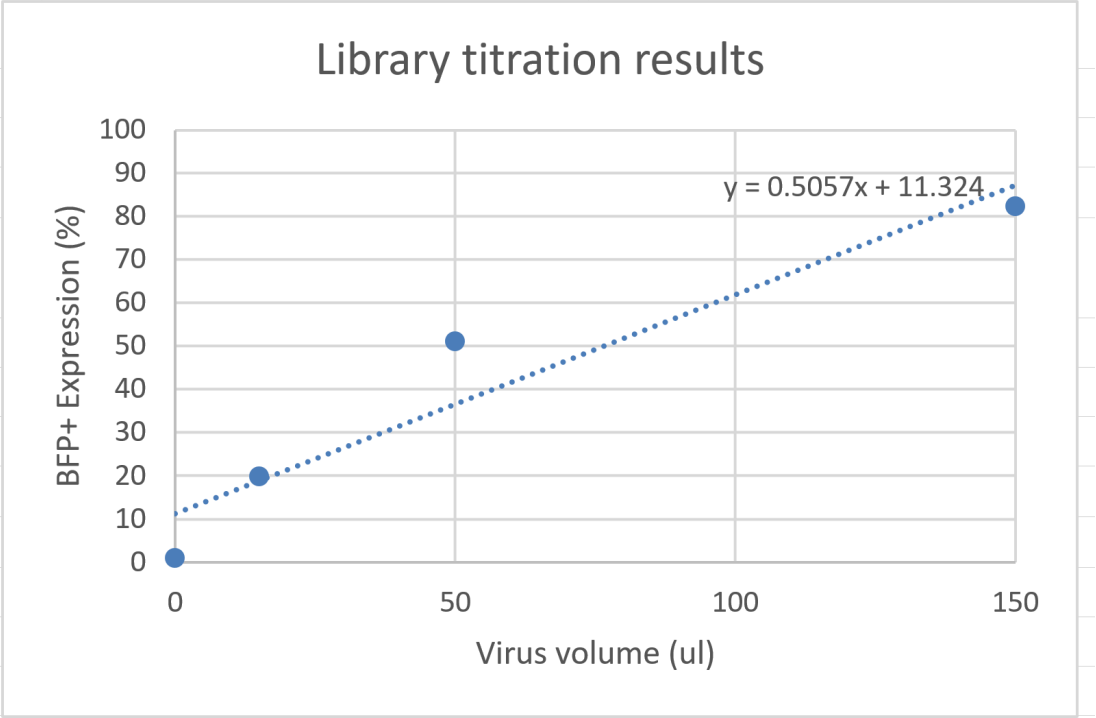


Figure 3. Example of transduction efficiency titration curve