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Puromycin titration of cancer cell lines

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Cellular Generation and P…

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

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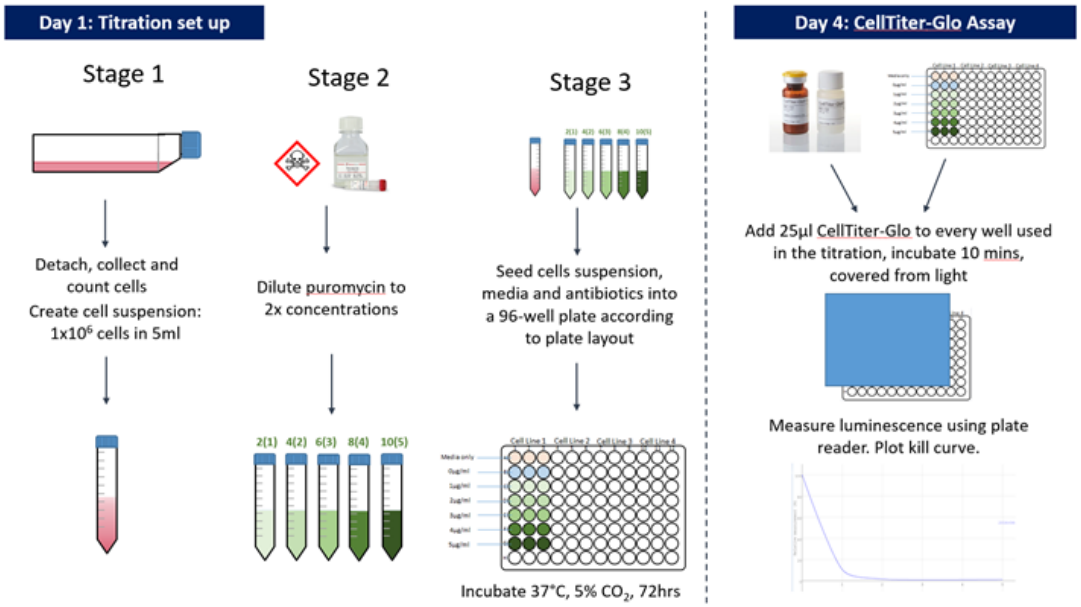
Abstract

This protocol is used to identify the most suitable puromycin concentration for the selection of Cas9 positive cancer cell lines transduced with guideRNA library virus.

Process diagram:



Guidelines

- It is important that the titration is carried out on the exact cells which will be used for the screen- e.g. the Cas9 version of the cell line.
- Ensure the cell suspension is mixed thoroughly to create an even single cell suspension before plating.
- 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 avoid ergonomic strain and to maintain homogenous solutions throughout.
- It is essential to use black 96-well plates in this protocol, as luminescence can carry over into neighbouring wells in clear plates.

Materials

MATERIALS

CellTiter-Glo(R) 2.0 Assay **Promega Catalog #**G9241

Falcon™ 15mL Conical Centrifuge Tubes **Fisher Scientific Catalog #**14-959-53A

TrypLE™ Express Enzyme (1X), no phenol red **Thermo Fisher Catalog #**12604021

Reagent Reservoir **Thermo Fisher Catalog #**9510047

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

Black walled 96 well plate **Fisher Scientific Catalog #**10419822

10mg/ml Puromycin **InvivoGen Catalog #**ant-pr-1

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

Equipment

Light Microscope
Microbiology safety cabinet (MSC)
Pipette Boy
Stripettes
Pipettes and tips
Centrifuge
Multichannel Pipette and tips
37 °C, 5%CO2 incubator
Plate reader

Safety warnings

- Puromycin is toxic if swallowed and harmful in contact with skin.
- CellTiter-Glo is harmful to aquatic life with long lasting effects.

Before start

- Pre-warm culture media to room-temperature.
- If necessary, prepare an aliquot of 1mg/ml puromycin (working concentration) by diluting a 10mg/ml stock 1:10 with sterile water.

Day 1: Titration plate set up

1 Detach, collect and count cells by following Steps 1-8 of the protocol: **Passaging adherent cancer cell lines**.

2 Resuspend 1x10^6 cells in 5 mL media, at a concentration of 2x10^5 cells/ml.

3 Using a 1mg/ml stock of puromycin, prepare five dilutions at 2x final concentration , by diluting the stock in media as shown in Table 1, column C & D. (When the 2x antibiotic concentration is diluted with an equal volume of cell suspension it will result in the final concentration shown in Table 1, column B).

Note

- Prepare a minimum of 5ml 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.

	2 x concentration (µg/ml)	Final concentration (µg/ml)	1mg/ml stock puromycin (µl)	Media (ml)	Total (ml)
	2	1	10	4.99	5.0
	4	2	20	4.98	5.0
	6	3	30	4.97	5.0
	8	4	40	4.96	5.0
	10	5	50	4.95	5.0

Table 1. Preparation of puromycin concentrations using 1mg/ml stock to achieve a 2x concentration.

Safety information

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

4 Pipette 75 µL cell suspension to the first 3 wells of rows B-G in a 96-well plate (row A is used a control with no cells, to subtract background luminescence).

Note

Always seed 3 wells per row as the titration is carried out in triplicate. Therefore, a 96-well plate can be used to titrate up to 4 cell lines at a time (see Fig. 1).

5 Pipette 150 µL media to the first 3 wells of row A, and 75 µL media to the first 3 wells of row B in a 96-well plate.

6 Pipette 75 µL of the puromycin 2x concentrations into the first 3 wells of rows C-G, to achieve the final concentrations as per the plate layout shown in Fig. 1.

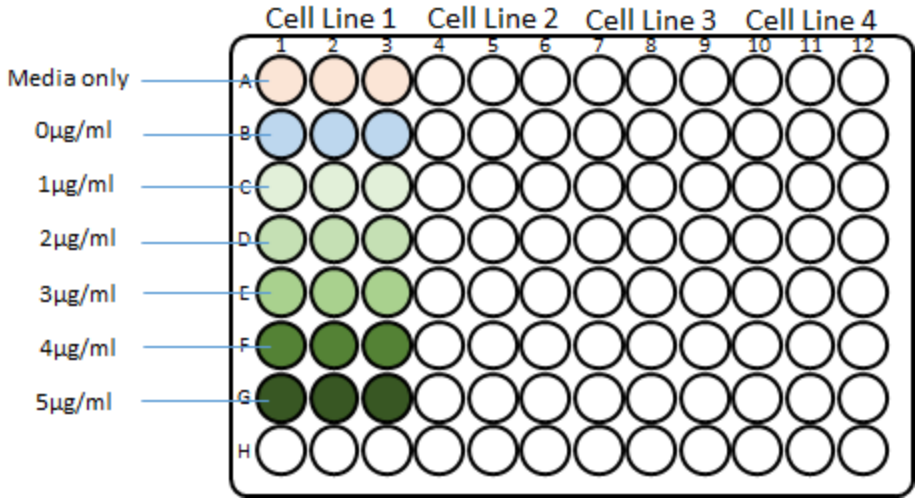


Figure 1. Plate layout for puromycin titration of one cell line.

7 Incubate at 37 °C , 5% CO_2 for approximately 72 hours.

Note

We use a testing time of 72 hours, but depending on the cell line, different time scales such as 24 or 48 hours could be used to pick the highest concentration.
Or for particularly resistance cell lines, the testing time can be extended to reflect the amount of time the cells will be in selection.

Day 4: Assessing cell viability using CellTiter-Glo

8 Thaw CellTiter-Glo 2.0 reagent and equilibrate to room-temperature prior to use. Mix by gently swirling to obtain a homogeneous solution.

Note

- The 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 stored in tin foil, and used in a cell culture hood with the light off where possible.

9 Remove the 96-well plate from the incubator and allow to equilibrate to room-temperature for 15 minutes.

10 Using a multi-channel pipette, add 25 µL CellTiter-Glo reagent to each well (1:6 dilution) and mix gently by rocking the plate back and forth. Incubate at room-temperature for 10 mintues (wrap plate in blue roll/foil or keep away from light where possible).

11 Use an appropriate plate reader to record the luminescence of each well.

Note

The plate reader should be set to an integration time of 1 second per well, and optimised for a peak emission wavelength of 560nm.

12 Create a kill curve as follows:
- 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 vaues.
- Divide the average luminescence for 1, 2, 3, 4 and 5µg/ml by the 0µg/ml average to get a relative percentage viability.
- Plot these values on a graph to create a kill curve.

The 'kill concentration' is the lowest concentration of puromycin which results in cell dealth of approximately 100% after 72 hours + 1µg/ml to account for difference in scale up.

For example, the 'kill concentration in Fig. 2 is 3µg/ml.

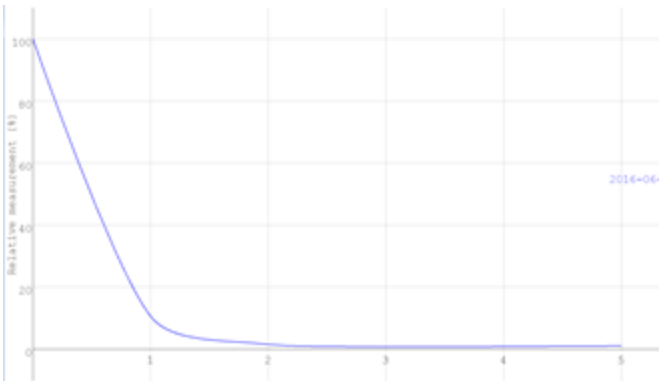


Figure 2. Kill curve for a puromycin titrated cancer cell line.