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Guide RNA Library Transduction of Cas9 Cancer Cell Lines

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1 Works for me dx.doi.org/10.17504/protocols.io.bg2njyde

Cellular Generation and Phenotyping

Verity Goodwin

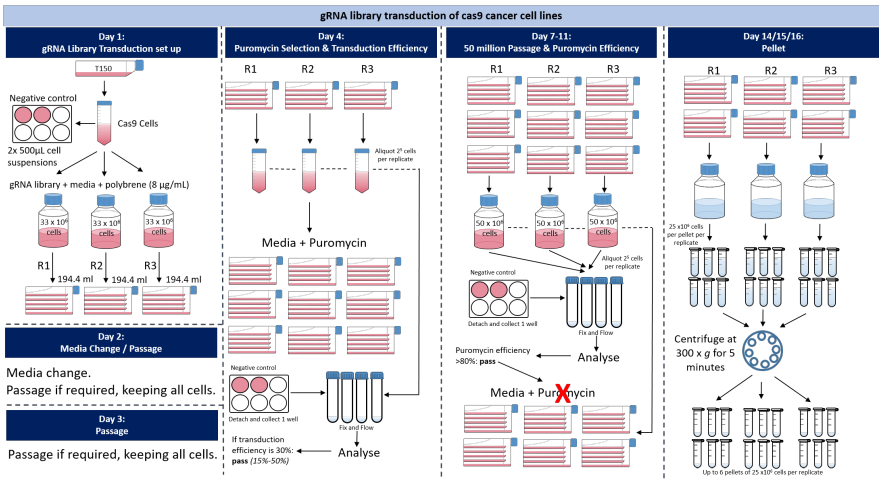
ABSTRACT

This protocol is for the whole-genome CRISPR screening of stably expressing Cas9 cancer cell lines in triplicate using 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 protocol can be followed assuming the following is known:
- The number of cells to be transduced
- The number of days required for screening
- How many cells to maintain throughout the screen
- The number of cells required per pellet
- The required coverage of the library

This protocol takes 16 days.

Process diagram:



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GUIDELINES

This library transduction is carried out in 33 x10^6 cells per replicate. These 33 x10^6 cells should occupy ~80% of the available surface area. Determine the surface area for each cell line as follows: Using previously collected data, estimate the surface area required for 33 x10^6 cells and select the smallest flask size that will accommodate 33x10^6 cells at 80% confluency.

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

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
Cell Culture Treated T150 Flasks	430825	Corning
Falcon Tissue Culture Treated 3 Layer Multi-Flask 525 Cm2	353143	BD Biosciences
Falcon Tissue Culture Treated 5 Layer Multi-Flask 875 Cm2	353144	BD Biosciences
Polybrene Infection / Transfection Reagent	TR-1003-G	Emd Millipore
DNA LoBind 2.0ml PCR Clean Eppendorf Tubes	0030 108.078	Eppendorf
Puromycin (10mg/ml)	ant-pr-1	InvivoGen
250ml Storage Bottle - Disposable With Plug Seal Cap Sterile 45mm neck	430281	Corning
Microcentrifuge tube Safe-Lock write-on 1.5mL Eppendorf Tube	0030 120.086	Eppendorf
Human Improved Genome-wide Knockout CRISPR Library	67989	addgene

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.

Equipment

- Microbiological Safety Cabinet (MSC)
- Centrifuge
- Microfuge
- Media dispensing pump (recommended)
- Pipetboy
- Stripettes
- P1000 pipette and tips

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- 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.	
Puromycin	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

- Know library volume from library titration
- 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: Transduction

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

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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.

3 Carry out each library transduction in triplicate. Prepare each replicate in a 250ml storage bottle as per Table 1 to transduce the cells at 100x coverage. Mix well, and seed into the appropriate labware. Clearly label each replicate (R1, R2, and R3).

It is recommended to use a media dispensing pump to dispense volumes greater than 50ml.

It is recommended to select the flask size for 33 million cells to comfortably fit for each cell line (see guidelines for more information).

Components	T150	T525	T875	2x T875s
Cells	33 x10^6	33 x10^6	33 x10^6	33 x10^6
Polybrene (µL)	8 µg/mL = 26.7	8 µg/mL = 93.33	8 µg/mL = 156.66	8 µg/mL = 313.32
Volume of virus library (mL)	(LT result x 15.6*) /1000	(LT result x 54.7*) /1000	(LT result x 91.1*) /1000	(LT result x 182.3*) /1000
Media (mL)	33.33 – (virus volume + cell volume + polybrene)	116.67 – (virus volume + cell volume + polybrene)	194.4 – (virus volume + cell volume + polybrene)	388.8 – (virus volume + cell volume + polybrene)
Total volume per flask (mL)	33.33	116.67	194.4	388.8

*Scaling Factor: This is the calculated based on the surface area of a 6 well plate (9.6cm^2) as this is the labware for the library titration, and the labware for the transduction.

Table 1. Table of components per replicate for transduction at 100x coverage for the gRNA library

4 Remove **1 mL** of stock cell suspension and seed into two wells of a 6 well plate (**500 µl** per well). Add **1.5 mL** media to each well. Place plate in the incubator. One well will be fixed on day 4 with a sample of transduced cells to act as a negative control to identify the transduction efficiency.

Maintain the second well for the remainder of the screen, passaging when required. This well will be fixed when puromycin selection efficiency is assessed to act as the negative control.

Day 2

5 Media change the flasks. Passage cells if required, keeping all cells (see protocol "Passaging adherent cancer cell lines", hyperlink in step 1).

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It is recommended to use a media pump to dispense volumes greater than 50ml.

Day 3

6 Passage cells if required, keeping all cells (see protocol "Passaging adherent cancer cell lines", hyperlink in step 1).

Day 4: Puromycin Selection

7 Detach and collect cas9 cells as per protocol "Passaging adherent cancer cell lines" steps 1-8 (hyperlink in step 1).

8 Take an aliquot of ~200,000 cells in a 1.5ml tube to test transduction efficiency using FACS.

The transduction efficiency for the library needs to be >15% to ensure a sufficient level of library representation. It should be ~30% for each replicate.

8.1 Detach and collect the negative control cells from the 6 well plate following steps 8-11 in the protocol "Library Titration of Cas9 Cancer Cell Lines" found here: https://protocols.io/view/guide-rna-library-titration-of-cas9-cell-lines-bgxujxnw.html

8.2 Fix the 4 cell suspensions following protocol "Fixing cell pellets" (R1, R2, R3, negative control) found here: https://protocols.io/view/fixing-cell-pellets-bg2fjybn.html

8.3 Carry out flow cytometry and FACS analysis following steps 16-20 in the protocol "Library Titration of Cas9 Cancer Cell Lines" (hyperlink in step 8.1).

9 Seed all remaining cells at a 1:3 split ratio (slow growing lines can be split 1:2) in appropriately sized labware (e.g.: if the cells were in 1x T875, re-seed all cells into 3x T875s) in the **presence of puromycin** at the concentration determined by the puromycin titration, see Table 2 for volumes.

It is recommended to use a media pump to dispense volumes greater than 50ml.
Puromycin is toxic if swallowed and harmful in contact with skin.

Size of Flask	Volume of Media (ml)	1µg/ml Puromycin (µl)	2µg/ml Puromycin (µl)	3µg/ml Puromycin (µl)	4µg/ml Puromycin (µl)
T525	50	60	120	180	240
T875	100	100	200	300	400

Table 2. Volume of 1mg/ml puromycin stock to add to each flask

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Day 7-11

10

It is important that at least **50 million cells** are retained at every passage going forward to maintain the integrity of the library as this maintains 5 times the original 100x coverage.

Inspect cells under a microscope. If cells are <90% confluent, media change each flask with fresh media plus puromycin.

11 If cells are >90% confluent, passage each replicate, and carry out FACS analysis to determine if puromycin selection has been successful:

11.1 Detach and collect cas9 cells as per protocol "Passaging adherent cancer cell lines" steps 1-8 (hyperlink in step 1).

11.2 Take an aliquot of ~200,000 cells in a 1.5ml tube per replicate.

11.3 Detach and collect the negative control cells from the 6 well plate following steps 8-11 in the protocol "Library Titration of Cas9 Cancer Cell Lines" (hyperlink in step 8.1).

11.4 Fix the 4 cell suspensions following protocol "Fixing cell pellets" (R1, R2, R3, negative control) found here: https://protocols.io/view/fixing-cell-pellets-bg2fjybn.html

11.5 Carry out flow cytometry and FACS analysis following steps 16-20 in the protocol "Library Titration of Cas9 Cancer Cell Lines" (hyperlink in step 8.1).

If puromycin selection has been successful, the proportion of BFP positive cells should be >80%

12 If puromycin selection was successful, re-seed a minimum of 50 million cells per replicate into an appropriate number of flasks, following protocol "Passaging adherent cancer cell lines" (hyperlink in step 1) **without** puromycin. The screen can be continued in the absence of puromycin.

13 If puromycin selection was unsuccessful, re-seed all cells into an appropriate number of flasks, following protocol "Passaging adherent cancer cell lines" (hyperlink in step 1) **in the presence of** puromycin.

7 days post puromycin selection, media change the cells **without** puromycin. The remainder of the screen can be continued in the absence of puromycin.

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14 Following puromycin selection, passage cells when >80% confluent, keeping a minimum of 50 million cells. If the cells are slow growing, keep a minimum of 100 million cells.

If by day 10, cells have not been passaged, media change the flasks without puromycin.

If the cells do not require passaging until the day of pelleting, follow steps 11.2 - 11.4 to determine the puromycin selection efficiency on the day of pellet.

Day 14/15/16: Pellet

15 Pellet cells when confluent, between day 14 and day 16.

Detach cells, collect, and count each replicate following protocol "Passaging adherent cancer cell lines" steps 1-8 (hyperlink in step 1).

Collect the cell suspensions in PBS at step 7.

16 Aliquot 25 x10^6 cells into up to 6x 2ml PCR clean/DNA LoBind tubes and centrifuge at **300 x g** for 5 minutes using a microcentrifuge in the MSC.

17 Carefully remove the supernatent using a P1000 pipette, ensuring not to dislodge the cell pellet. These cell pellets can be stored in a **-80 °C** freezer until DNA extraction is required.

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