SUPPORT PROTOCOL 2

BIOLOGICAL TITRATION OF LENTIVECTORS USING FLOW CYTOMETRY

This method can only be used to titer stocks of vectors that carry a transgene which is easily monitored by flow cytometry (such as GFP, or other living colors, or any membrane protein that can be detected by flow cytometry), and whose expression is governed by a promoter that is active in HCT116 cells (tissue-specific promoter-containing vector must be functionally assayed in specific cells, and titered by QPCR in HCT116 cells; see below). This protocol describes the titration of a PGK-GFP vector (pRRL, also see Basic Protocol).

Materials

HCT 116 cells (ATCC cat. no. CCL-247)

_HCT116 is a subclone of HT1080 cells. These cells are human cells and are commonly used as they are highly permissive to lentiviral vector-mediate transduction. Furthermore, their genome is more stable than that of HeLa cells allowing a more accurate titration by QPCR (see support protocol 3)._ 

Dulbecco’s modified Eagle medium/10% FBS (DMEM-10; APPENDIX 2A)

Lentiviral vector sample for titration, carrying GFP transgene (see Basic Protocol)

Phosphate-buffered saline (PBS), pH 7.4 (APPENDIX 2A)

0.05% trypsin/EDTA (e.g., Invitrogen)

1% (w/v) formaldehyde: dilute 1 ml of 37% formaldehyde (Sigma) in 36 ml PBS (APPENDIX 2A); store at 4°C

12-well tissue culture plates (e.g., BD Biosciences)

Fluorescence-activated cell sorter (FACS, Becton Dickinson; with 488 nm excitation laser and green filter) or equivalent flow cytometer, and appropriate tubes

**NOTE:** All solutions and equipment coming into contact with living cells must be sterile, and aseptic technique should be used accordingly.

**NOTE:** All culture incubations should be performed in a humidified, 37°C, 5% CO₂ incubator unless otherwise specified.

Perform assay

1. On day 1, plate around 1 to 5.10⁵ cells/well in a 12 wells plate with 1 ml of DMEM-10/well. Make sure that the cells are well separated and uniformly distributed in the well.

   _On average: starting from one confluent 10cm dish, plate 1/15 per 12 wells plate._

2. On day 2, use 1 well to count the cells. It should be between 1 to 4.10⁵ cells/well. Remove medium.
For non concentrated vector transduce the cells with: 500μL, 100μL, 50μL, 20μL and 10μL of crude supernatant. Complete the volume to 500μL with DMEM-10. Keep one well as non transduced control (NI). Perform each transduction in duplicate.

For concentrated vector transduce the cells with: 1μL, 10⁻¹, 10⁻², 10⁻³ and 10⁻⁴ μL of vector in 500μL of fresh DMEM-10.

Polybrene can be omitted for transduction with VSV-G pseudotyped vectors since this compound does not influence permissivity of cells to VSV-G pseudotyped vectors.

3. On day 5, wash the cells with 1 ml PBS, detach them by adding 200 μL of trypsin/EDTA per well and incubate 1 min at 37°C.

4. Add 800 μL of DMEM-10 and mix well to resuspend the cells. 

   This step inactivates the trypsin and EDTA.

5. Transfer the cells into a 5-ml FACS tube and centrifuge 5 min at 500 g 4°C. Remove the supernatant.

6. Resuspend the pellet in 500 μL of 1% formaldehyde in PBS and incubate 5 min at room temperature.

   This step will fix the cells and inactivate the vector particles. Samples can thus be taken out of the P2 laboratory at this point. Be aware that some proteins are sensitive to formaldehyde such as dsRed and dTomato and will no more be fluorescent after fixation.
7. Wash cells with PBS 1X, centrifuge 5 min at 500 g 4°C and resuspend the pellet in 1 ml PBS1X.

8. Analyze cells for GFP expression using FACS or equivalent flow cytometer.

*Cells fixed with formaldehyde can be stored in the dark at 4°C for several hours. A final 0.5% formaldehyde concentration is enough to fix cells and inactivate vectors. Increasing formaldehyde concentration (up to 4% final) will increase the autofluorescence of cells and decrease GFP fluorescence.*

**Calculate titer**

10. Chose dilutions yielding 1% to 20% GFP positives for titer calculations.

*In a typical titration experiment, only dilutions yielding to 1% to 20% of GFP-positive cells should be considered for titer calculations. Below 1%, the FACS may not be accurate enough to reliably determine the number of GFP-positive cells. Above 20%, the chance for each GFP-positive target cell to be transduced twice significantly increases, resulting in underestimation of the number of transducing particles.*

11. Calculate titer using the following equation:

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\text{Titer (HCT116 transducing units/ml)} = \frac{\text{Number of target cells (count at day 1)} \times \frac{\% \text{ of GFP-positive cells}}{100}}{\text{Volume of supernatant in (mL)}}
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