DETERMINING VIRAL TITERS VIA FLOW CYTOMETRY:

REAGENTS:
- Polybrene
- PBS
- 100% Methanol (***Note: you can use Ethanol instead if you can't get Methanol***)

MEDIA:

Normal Media
DMEM
10% Heat inactivated FCS
1% L-glutamine (2 mM)
1% Penicilin (50 units/ml) + Streptomycin (50 µl/ml)

Infection Media
DMEM
2% Heat Inactivated FCS
1% L-glutamine (2 mM)

EQUIPMENT:
- 6 well tissue culture grade plates
- FACS tubes
- Tissue culture incubator at 37ºC/5% CO_2
- Fluorescence-aided cell sorting (FACS) capabilities

CELL LINE: HT-1080

PROTOCOL:

Day 1) Prepare Cells:
- Lift exponentially growing HT-1080 cells & count them
  - ***Note: Cells shouldn't be more than 70% confluent when you lift them***
- Seed 100,000 cells in normal growth media (total volume = 2 ml) into each well
- Swirl the plate to spread cells evenly around the well
- Incubate at 37°C/5% CO_2 overnight to allow cells to adhere

Day 2A) Prepare virus:
- Label eppendorf tubes (from 1 to 6)
- Add the following reagents to the eppendorf tube, in the order shown below:
  - 8 µg/ml Polybrene
  - Infection media
  - Virus sample

  Note: Each tube should have a total volume of 500 µl
- Mix contents by inverting the tube 6 times

Day 2B) Infect Cells:

- Remove media from cell
- Wash (x1) gently with PBS
- Remove PBS
- Add 500 µl of infection media containing virus to each well (refer above) as per the template below

6-well Plate Template:

100,000 HT-1080 cells 8 µg/ml Polybrene 20 µl virus # 1 Infection media (TOTAL VOL = 500 µl)	100,000 HT-1080 cells 8 µg/ml Polybrene 10 µl virus # 1 Infection media (TOTAL VOL = 500 µl)	100,000 HT-1080 cells 8 µg/ml Polybrene 5 µl virus # 1 Infection media (TOTAL VOL = 500 µl)
100,000 HT-1080 cells 8 µg/ml Polybrene 2 µl virus # 1 Infection media (TOTAL VOL = 500 µl)	100,000 HT-1080 cells 8 µg/ml Polybrene Infection media (TOTAL VOL = 500 µl) **No virus**	100,000 HT-1080 cells Infection media (TOTAL VOL = 500 µl) **No polybrene or virus**

- Swirl the plate gently (every 5 min for 30 min) to ensure all cells are coated with virus/media
- Incubate at 37°C/5% CO_2 overnight

Day 3) 24 hr After Infection (add media):

- Add 1.5 ml 'normal growth media' to each well

Note: total volume per well should now be 2 ml

Day 4A) FACS:

- Label 15 ml Falcon tubes (1-6)
- Collect media into a 15 ml falcon tube
- Add 1 ml PBS to each well
- Collect PBS & add to media in the 15 ml falcon tubes
- Add 1 ml Trypsin/EDTA to each well
- Incubate at 37°C/5% CO_2 for 5 min
- Check cells are in suspension.
    - If they are still attached, give the plate a light tap & check again.
    - If still attached, then incubate at 37°C/5% CO_2 for an additional 2-5 min
- Add 1 ml 'normal growth media' to each well
- Collect cells/media & add to the contents of the 15 ml falcon tubes
- Spin tubes at 1200 rpm for 5 min
- Remove supernatant
- Re-suspend cells in 1 ml PBS & transfer to labeled eppendorf tubes
- Spin at 11,000 x g for 1 min
- Remove PBS
- Re-suspend cells in 100 µl of 100% methanol
- Incubate at -20°C for 2 hr (to fix cells)
- Add 900 µl of PBS to each eppendorf tube
- Spin at 11,000 x g for 1 min
- Remove PBS/methanol

- Re-suspend cells in 500 µl of PBS
- Spin at 11,000 x g for 1 min
- Re-suspend cells in 300 µl of PBS & transfer to pre-labeled FACS tubes
- Check % cells that are GFP positive via flow cytometry

NEGATIVE CONTROLS:
No Virus
No Polybrene or Virus

NOTE: If the "no virus" sample has a different FACS profile than the "no polybrene or virus" sample, then you must use the "no virus" sample as your negative control for gating.

If however, there is no difference between the two negative control samples, then for all future experiments, you should prepare a "no polybrene or virus" sample only for your negative control so that we reduce the amount of polybrene we use.

Day 4B) Calculating Titers:
The following formula should be used to determine titer (TU) per ml.

5. Calculate the titer in transducing units (TU)/ml, according to the formula:

$$\frac{(1 \times 10^5 \text{ seeded cells} \times \% \text{ GFP-positive cells}) \times 1000}{\mu l \text{ of vector}}$$

For excel, prepare your spreadsheet as per the following example:

Cell	A	B	C	D	E
1	Virus Vector Name:	Number HT-1080 cells seeded	Volume of Virus vector used	% GFP Positive Cells	TU/ml
2	Virus # 1	100000	20	40.6	=((B2*D2)*1000)/C2
3	Virus # 1	100000	10	20.1	=((B3*D3)*1000)/C3
4	Virus # 1	100000	5	10.5	=((B4*D4)*1000)/C4
5	Virus # 1	100000	2	4.2	=((B5*D5)*1000)/C5

i.e. You want excel to complete the calculations in the following steps
Step 1) Calculate: "1 x 105 seeded cells x % GFP-positive cells"
i.e. 100000 x 40.6 = 4060000
Step 2) Calculate: "Answer for Step 1 x 1000"
i.e. 4060000 x 1000 = 4060000000 (or 4.06×10^9)
Step 3) Calculate: "Answer for Step 2 / µl of vector"
i.e. $4.06 \times 10^9 / 20 = 203000000$
Titre (TU) = 2.03×10^8 per ml