This protocol was adapted from Dr. Bruce McManus's group at University of British Columbia and was used in the following publication: Jensen KJ, Garmaroudi FS, Zhang J, Lin J, Boroomand S, Zhang M, Luo Z, Yang D, Luo H, McManus BM*, and Janes KA*. (2013) An ERK–p38 subnetwork coordinates host-cell apoptosis and necrosis during coxsackievirus B3 infection. *Cell Host Microbe*, 13, 67-76.

I. Preparation of live CVB3

1. Grow permissive "HeLa-CVB3" cells to ~90% confluency in a T-75 flask.
   - *HeLa-CVB3 is a variant of HeLa cells obtained from the McManus lab that is readily infected by CVB3; HeLa cells from ATCC do not work as well*
   - *Be sure to spray down the tissue-culture hood thoroughly with Cavicide before and after handling live virus*
2. Aspirate growth medium and wash with 10 ml PBS.
3. Thaw earlier CVB3 viral stock on ice.
   - *CVB3 is a BSL2-level pathogen that is particularly dangerous to fetuses, newborns, and young children*
   - *As a safety precaution, our lab does not allow expecting parents or parents with young children to handle the live virus*
4. Add 10^8 plaque-forming units (pfu) of CVB3 in 3 ml serum-free DMEM.
5. Incubate for 1 hr at 37ºC, gently shaking the flask every 10 min during the incubation.
6. Add an additional 3 ml serum-free DMEM + pen/strep (no serum) to the plate and incubate for 48 hr at 37ºC.
   - *Do not remove the earlier CVB3-containing medium*
7. Collect the 6 ml medium from the plate and transfer to a conical tube.
8. Remove dead HeLa cells from the virus stock by centrifuging at 5000 rpm for 5 min on a swinging-bucket centrifuge at 4ºC.
9. Aliquot the viral stock into screw-top tubes and store at –80ºC.
   - *Viral titers will drop a few-fold with each freeze-thaw cycle, so it is important to keep this in mind when performing infections at a defined multiplicity of infection (moi)*

II. Titering CVB3 viral stocks by plaque assay

1. Plate 10^6 permissive "HeLa-CVB3" cells per well of a 6-well dish overnight.
   - *Cells should be 95–100% confluent on the day of infection*
   - *The HeLa-CVB3 variant is essential for forming nice plaques with this assay*
   - *Each well allows one specific titer of virus to be tested, so scale up the plating if many viral stocks are to be tested*
2. Thaw viral stock(s) on ice.
3. Wash cells twice with serum-free media and then return plates to the incubator while serial dilutions of the virus are prepared.
4. Prepare serial dilutions of the viral stock in a 96-well plate while working in a tissue-culture hood:
   - 240 μl serum-free DMEM + 60 μl viral stock (mix 15× by pipetting up and down)
   - 270 μl serum-free DMEM + 30 μl solution A (mix 15× by pipetting up and down)
   - 270 μl serum-free DMEM + 30 μl solution B (mix 15× by pipetting up and down)
   - 270 μl serum-free DMEM + 30 μl solution C (mix 15× by pipetting up and down)
   - 270 μl serum-free DMEM + 30 μl solution D (mix 15× by pipetting up and down)
   - 270 μl serum-free DMEM + 30 μl solution E (mix 15× by pipetting up and down)
   - 270 μl serum-free DMEM + 30 μl solution F (mix 15× by pipetting up and down)
   - 270 μl serum-free DMEM + 30 μl solution G (mix 15× by pipetting up and down)
   - *Change tips in between dilutions to prevent virus carryover*
   - *Prepare further dilutions if necessary depending on expected concentration of virus. Dilution H is usually sufficient for measuring titers around 10^9 pfu/ml*

- *If higher titers are expected, you may not include the first few dilutions as they will not be informative for calculating pfu/ml*

5. Aspirate the medium from the 6-well plate(s) and add 200 μl of each virus dilution from Step #3.
   - *Be sure to include a "no virus" control well on the plate that simply receives 200 μl serum-free DMEM*
6. Rock the plate to ensure equal coverage of the virus, and incubate for 40 min at 37ºC, gently rocking the plate(s) every 10 min.
7. During the 40-min incubation, autoclave a 1.5% (w/v) solution of agar in water (liquid cycle, 121 ºC/ 250 ºF).
   - *Depending on the amount of agar being used, it may be necessary to keep agar in a hot water bath to prevent solidification before use.*
8. At the end of the 40-min incubation, aspirate the virus solution, wash cells with serum-free DMEM.
9. Mix one part 1.5% agar solution with one part 2× DMEM growth medium (2 ml per well).
10. Aspirate the serum-free DMEM from the wells and add 2 ml of the agar-DMEM mixture to each well.
11. Allow the agar-DMEM mixture to solidify within the wells at room temperature (~15 min).
12. Return the plate to the 37ºC incubator and wait 48–72 hr.
13. Fix the plates by adding 2 ml Carnoy's fixative (3 parts EtOH + 1 part acetic acid) per well.
14. Incubate for 30 min at room temperature.
15. Decant the fixative and use a small pliable weighing spatula to detach the agar from the sides of the well. With the plate upside down, use the end of the spatula to gently pull an edge of the agar plug away from the plate. The plug should slowly fall out of the plate on its own, which can be encouraged by gentle tapping on the bench top or with the spatula on the drooping edge of the agar.
    - *Be very careful at this step to avoid detaching adherent HeLa cells from the plate surface*
    - *Stabilizing both arms on the benchtop can decrease the frequency of scratches.*
16. Add 1 mL of 0.5% (w/v) crystal violet to each well after removal of the agar.
    - *Be sure to collect crystal violet after staining for proper disposal as hazardous waste*
    - *Be very careful not to scratch the wells when removing crystal violet*
17. After crystal violet stain is removed, rinse the entire plate under running water to visualize the plaques.
    - *Plaques will appear as clear foci on a purple background of remaining viable HeLa cells*
18. Scan the plate wet on an suitable scanner (i.e. Odyssey). Choose an appropriate resolution (42 μm is optimal for high-resolution imaging). Use the scan to count the number of plaques per well to calculate the viral titer (pfu/ml)
    - One should expect viral titers in the range of 10^8–10^{11} pfu/ml
19. To calculate pfu/ml:
    pfu/ml = (# of plaques in well)*(well volume to overlay volume ratio)*(10^{well-1})*(stock volume to sample volume ratio)

    For example:
    4 plaques counted in 5th well (Dilution E):
    =(4 pfu)*(300 µl /200 µl)*($10^{(5-1)}$)*(1000 µl /60 µl)
    =1,000,000 pfu/ml

Buffer recipes

- **1x HeLa growth medium** Store at 4ºC
  500 ml High-glucose DMEM with L-glutamine (Gibco #11965-092)
  5 ml 100× penicillin-streptomycin (Gibco #15140-122)
  50 ml FBS

- **Serum-free DMEM** Store at 4ºC
  500 ml High-glucose DMEM with L-glutamine (Gibco #11965-092)
  5 ml 100× penicillin-streptomycin (Gibco #15140-122)

- **2× DMEM growth medium** Store at 4ºC
  1 packet DMEM powder (Gibco #12100-046)
  3.7 g Sodium bicarbonate
  400 ml ddH_2O
  100 ml FBS
  5 ml 100× penicillin-streptomycin (Gibco #15140-122)
  To make 2× media, first dissolve DMEM powder in 350 ml ddH_2O. Add 3.7g of sodium bicarobonate then pH to 7.4. Media can then be volumed up to 400 ml with ddH_2O. Sterile filter the media through a 0.22-μm pore filter in a tissue culture hood. Add sterile FBS and penicillin-streptomycin.

- **0.5% crystal violet solution:**
  0.5 g crystal violet
  75 ml ddH_2O
  25 ml 100% methanol
  After preparation, pass through a 0.45 μm filter to remove particulates.

Cardiovascular Research Laboratory

Virus Titre Calculation

1 000 000 Virus particles in 1000 ul

(1x10^6 pfu/ml)

Sample volume

60ul = 60 000 virus particles.

30ul = 30 000 Virus particles

$\frac{60ul}{1000ul} \times \frac{1\,000\,000\ pfu}{1000ul} = 60\,000\ particles$

30ul x $\frac{1\,000\,000\ pfu}{1000ul}$

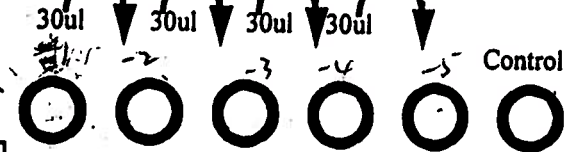
= 30 000 particles

30+270ul

(30)

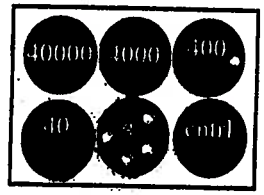
dilute 60ul of sample into 1st well

96 Well Plate Dilution Series
(Dilute in MEM without FBS)



Dilutent volume	240ul	270ul	270ul	270ul	270ul	270ul
# pfu/well after dilution	54 000 in 270ul	5400 in 270ul	540 in 270ul	54 in 270ul	6 in 300ul	
# pfu/ul	200/ul	20/ul	2/ul	0.2/ul	0.02/ul	
overlay to well volume ratio	200/270	200/270	200/270	200/270	200/300	

Overlay 200ul of each dilution sample to appropriate well



Plaques

count plaques @ well # 4
plaques @ well # 5

Calculate pfu/ml

= (# plaques in well)(well volume to overlay volume ratio)(10$^{(well\#-1)}$)(stock volume to sample volume ratio)

= (4 pfu) (300ul/200ul) ($10^{(5-1)}$) (1000ul/30ul)

= 1 000 000 pfu/ml.

Calculate pfu/mg

Tissue mass (mg) = (tissue + tube mass) - (tube mass) ** Remember that mass of individual tubes vary

Homogenize tissue in 500ul MEM without FBS

$$= \frac{(\#\ plaques\ in\ well)(well\ volume\ to\ overlay\ volume\ ratio)(10^{well\#-1})(\ homogenization\ volume\ to\ sample\ volume\ ratio)}{(mg\ tissue)}$$

2% AA w/ Eth