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## Virus Plaque Assay Protocol

This is our preferred protocol which we use routinely at VIRAPUR to perform plaque titration and agarose overlay assays. The technique is applicable to multiple virus systems, and we have used it successfully with many human and animal cytopathic viruses. If you want to outsource this assay, contact [VIRAPUR](http://www.virapur.com). Information and pricing can be obtained by calling (858) 824-9000 or [email us](mailto:info@virapur.com) to arrange this testing. Send our viral experts your samples to be titered and we will generate answers or deliver isolates within 2-3 weeks.

### DEFINITIONS:

- **Plaque:** an area of cells in a monolayer which display a cytopathic effect, *e.g.* appearing round and darker than other cells under the microscope, or as white spots when visualized by eye; the plaque center may lack cells due to virus-induced lysis.
- **Plaque-forming unit (PFU):** a virus or group of viruses which cause a plaque.

### MATERIALS AND EQUIPMENT

- Cell Growth Media (DME high glucose with 4 to 6 mM glutamine and 10% fetal bovine serum); Pen-Strep and Fungizone can be added if desired
- Plaquing Medium (DME high glucose with 4 to 6 mM glutamine and 2% fetal bovine serum)
- PBS, no Ca or Mg
- Agarose, UltraPure (Gibco Cat. No. 15510-019 or equivalent)
- Tissue culture grade water (WFI)
- Eppendorf or similar pipettors capable of dispensing 10 to 100  $\mu$ l
- Tubes to make your dilutions
- 65°C water bath
- Microwave oven
- Vortex
- Various tissue culture grade sterile bottles of appropriate size (100-250 ml)
- MTT (Sigma, M2128)
- 6 well tissue culture dishes
  - Certain cell lines such as 293 are very sensitive to manipulation and pH and may require dishes pre-coated with Collagen. Such dishes are commercially available from Biocoat, Falcon

### HARVEST AND PLATING OF CELLS IN 6-WELL DISHES

1. Make a solution of cells at  $1 \times 10^6$  cells per ml in Growth Media.
2. Add 2.0 ml of growth media with cells to each well. Jiggle the plate to evenly distribute cells, 12 o'clock to 6 o'clock and 9 o'clock to 3 o'clock (left-right, top-bottom). Incubate plates in a 37°C, CO<sub>2</sub> incubator.
3. After ~12-24 hours, the cells should be ~90-100% confluent. Go on to the next step immediately. Waiting several more days for cells to become completely confluent is not optimal.

## **VIRUS DILUTION AND INFECTION**

1. For adenovirus, when using unknown concentrations of virus directly from infected cell culture, supernatant plus lysed cells should exhibit a titer of  $10^8$  through  $10^9$  PFU/ml. Optimal levels of purified virus will have a titer from  $10^9$  through  $10^{10}$  PFU/ml.
2. Other viruses will vary in titer. See the table below for expected titers of various viruses.
3. Prepare dilutions of virus in PBS or DMEM. Never make more than a 100-fold dilution; always pipette at least 10  $\mu$ l of virus or solution to reduce pipetting error.

How we usually dilute virus at VIRAPUR:

1. Prepare 4 tubes; each tube will contain 2 ml of PBS.
2. Add 20  $\mu$ l of virus sample to the first tube. Vortex.
3. Repeat the dilution process through all four tubes.

The tubes will now have these effective dilutions of virus:  $10^{-2}$  (1/100),  $10^{-4}$  (1/10,000),  $10^{-6}$  (1/1,000,000),  $10^{-8}$  (1/100,000,000).

## **INFECTION OF THE MONOLAYERS**

1. Pipette off and discard ONE ML of media from each well. One ml of media should now remain on each monolayer.
2. Add 100  $\mu$ l or 10  $\mu$ l of each dilution in duplicate to each well, letting the virus flow gently into the media.

[Consider this: If 100 $\mu$ l of a  $10^6$  dilution yields 25 plaques, the titer of virus is  $2.5 \times 10^8$  PFU/ml. If 10 $\mu$ l of a  $10^6$  dilution yields 30 plaques, the titer of virus is  $3.0 \times 10^9$  PFU/ml.]

3. Incubate the infected monolayers at 37°C for four to sixteen hours; mildly shake the plates gently several times during this adsorption period.

## **AGAR OVERLAY**

1. Prepare a sterile solution of 4% agarose in dH<sub>2</sub>O by autoclaving at 121°C for 20 minutes. Agarose may be stored on the shelf at room temp or used immediately after equilibrating in a 65°C water bath. Alternatively, 100 ml aliquots of solidified agarose can be melted in a microwave for about 1 minute and cooled to 65°C in a water bath. Agarose can burn in the autoclave, so don't leave the agarose solution in a hot autoclave overnight.
2. Warm the plaquing media (see above, 2 ml per well to be overlaid) in a 37°C water bath until equilibrated. Make absolutely sure your media is at 37°C.
3. Gently draw media out of each Ad5 infected monolayer well and discard. We suggest an aspirating pipette attached to a vacuum.
4. Mix the volume of media you need ( i.e. 5 plates, 30 wells, 60 ml) in a 37°C pre-warmed container and add 0.11 volumes of liquid agarose to the bottle with swirling (1:10 dilution). Shake vigorously to mix. Do not be afraid to mix well and immediately.
5. With a new pipette, immediately but gently add 2 ml of the agarose/growth media mixture to each well, pipetting it down the side of the well.
6. Let the plate(s) sit for 15 minutes in the level hood at RT as the agar overlay turns solid.
7. Move the plate(s) to a humidified incubator at 37°C and 7.5 to 10% CO<sub>2</sub>. Some viruses require different temperatures.

## PLAQUE VISUALIZATION

- Plaques will be visible by day 5 to 7 after infection, and if you desire you can stain the monolayer to visualize and count the plaques on the final day of plaque development. With the naked eye, look for white dots on the monolayer. These dots may be more easily visualized by viewing the plate with oblique light. Note: It is critical to confirm that the dots are plaques by inspection under a microscope.
- If desired, stain the plate(s) at 5-6 days post infection or when plaques have fully developed. Stain for 3 hr at 37°C by adding 0.1 volume of MTT solution (5 mg/ml in PBS) (Sigma, M2128). You can pick viable virus plaques through the MTT staining. Sterilize the MTT solution by filtration if plaques will be picked. The monolayer will appear blue/black and plaques will be clear areas. This method of staining gives much clearer plaques than neutral red staining.

Virus	Cell Line	Expected Titer of Crude Virus (pfu/ml)	Days of culture until Plaques Visible
Adenovirus	293 for Recombinants A549 for Wild Type	$10^8$ to $10^9$	7-10
Herpesvirus	Vero	$10^7$ to $10^8$	3-5
Influenza	MDCK	$10^6$ to $10^8$	3-5
Rhinovirus	H1 HeLa	$10^7$ to $10^8$	5-7

## PFU/ml CALCULATION

- After counting plaques, you can calculate the concentration of the initial viral suspension in PFU/ml.
- Take the dilution of virus you plated and the volume of virus solution you placed on the single monolayer to determine the PFU/ml.
- Example: If you placed 10  $\mu$ l of a  $10^{-6}$  dilution on a well and you see 44 plaques, the titer from that well is  $4.4 \times 10^9$  PFU/ml; your initial virus suspension is  $4.4 \times 10^9$  PFU/ml.
- Example: If you placed 100  $\mu$ l of a  $10^{-8}$  dilution on a well and you see 88 plaques, the titer from that well is  $8.8 \times 10^{10}$  PFU/ml.
- Perform the appropriate statistical calculations if you like. At VIRAPUR, we find that this assay is consistent within about a factor of 2 to 3!

## We offer a Collagen Coating procedure:

### COLLAGEN COATING PLATE PROCEDURE

1. Prepare a solution of 0.5 mg/ml collagen (Celltrix, Vitrogen 100) in 1 mM acetic acid. Prepare 6 ml of this solution per six-well plate to be used.
2. Filter the solution through a 0.22 micron filter.
3. Add 1.0 ml of collagen solution to each well. Incubate for at least 30 minutes at RT.
4. Using sterile technique, pipette off and save the collagen solution for future use. You can use the plate(s) immediately or you can let the plate(s) dry covered in a sterile environment overnight. When dry, recover the plates and use immediately or reseal in plastic bags and store at room temperature until use.