This method is based on that published here:(1)

1.**Bewig, B. and W.E. Schmidt.** 2000. Accelerated titering of adenoviruses. BioTechniques *28*:870-873.

Day 1

Seed a 12-well plate with 293TREx cells - 5×10^5 cells/well in 1ml total volume & allow them to adhere overnight.

> Note. You may need to adjust this up or down a bit depending on your cells/counting etc. They should be almost confluent, but it's very important they are not overconfluent. If they are overconfluent then you will get no staining atall.

Day 2

Prepare serial dilutions of your viral stock. Dilutions of 10^{-4} & 10^{-5} are usually suitable.

> i.e. Dilute virus stock 10µl in 990µl (10^{-2} dilution), then dilute that 10 in 990µl (10^{-4}), then dilute that 100 in 900µl (10^{-5})

Add 100µl of the 10^{-4} dilution to a well, and 100µl of the 10^{-5} dilution to another well.

> For increased accuracy, 2 wells should be infected with each duplicate and plaque counts averaged at the end.

Wait 48 hours

Day 4

1. Aspirate medium and allow wells to air dry. No need to wash with PBS.
2. Gently add 1ml ice cold 50/50 acetone/methanol and incubate at -20°C for 10 mins
3. Aspirate acetone/methanol (the monolayer is much less likely to be dislodged now) and wash 3 times in PBS with 1% BSA
4. Dilute chemicon goat anti-Adenovirus primary antibody (cat. no. AB1056) (1 in 5,000 dilution in PBS+1% BSA) & add 0.5 ml to each well.
5. Rock at 37°C for 1 hour.
6. Aspirate antibody & wash each well 3 times in PBS+1% BSA.
7. Dilute secondary antibodies (anti-goat hrp) (donkey anti-goat & chicken anti-goat from abcam) 1 in 1,000 each in PBS+1%BSA & add 0.5 ml to each well
8. Rock at 37°C for 1 hr.
9. Aspirate antibody & wash each well 3 times in PBS+1% BSA.
10. Make up metal enhanced DAB substrate (cat. no. 34065 from pierce):
    1. Remove the DAB/Metal Concentrate (10X) from -20°C storage and mix well by inverting the bottle. Remove quantity required for use and immediately return bottle to -20°C. Do not allow it to reach room temp.
    2. Prepare a 1X working solution of the DAB/Metal Concentrate (10X) by adding the Stable Peroxide Buffer and mixing well. For example, if 5 ml of substrate is required, add 4.5 ml of the Stable Peroxide Buffer to 500 µl of the DAB/Metal Concentrate. The 1X substrate solution is stable for several hours at 4°C.
11. Add 500µl DAB substrate to each well & incubate 10mins at room temp.

12. Remove DAB metal substrate & add 1 ml PBS to each well (Note you may find you can't see the brown staining in the centre of the well. Just count fields from around the edges if this happens).

Count cells & calculate pfu/ml

Count the number of positive wells in 3 fields of view. Try & use an objective & dilution that gives 5-50 positive cells/field. Calculate the average number of positive cells/well.

Use the below table to determine the number of fields/well:

Objective Lenses	Fields/Well		
	12-Well Plate	24-Well Plate	96-Well Plate
4X	19	10	1.6
5X	30	16	2.6
10X	150	79	12.6
20X	594	313	50

Calculate the plaque forming units/ml (pfu/ml) as:

$$\frac{(Infected\ cells\ /\ field) \times (fields\ /\ well)}{volume\ virus(ml) \times (dilution\ \ factor)}$$

Or use the Excel spreadsheet to do it all for you.