Viral Assays: Physical and Infectivity Assays

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Virus Assays: Qualitative/ Quantitative

- Viral quantification: Counting the number of viruses in a specific volume to determine virus concentration
- Requirement
  - the production of viral vaccines,
  - recombinant proteins using viral vectors and viral antigens all require virus quantification to continually adapt and monitor the process in order to optimize production yields
  - Clone screening
  - MOI (Multiplicity of Infection-ratio of phage to host) optimization for infectious biology assays, drug testing, immunopathogenesis assay, dose response curves

Traditional Method

- Transmission Electron Microscopy
- Plaque Assay
- Endpoint Dilution Assay
- Serological/Protein Assays
  - Hemagglutination assay
  - Complement fixation assay
  - Radio Immuno Assay
  - ELISA
  - Immunofluorescence
- Nucleic Acid methods:
  - Hybridisation
  - PCR

Electron Microscopy

10^6 virus particles per ml required for visualization, 50,000 - 60,000 magnification normally used. Viruses may be detected in the following specimens.

Faeces	Rotavirus, Adenovirus
	Norwalk like viruses
	Astrovirus, Calicivirus
Vesicle Fluid	HSV
	VZV
Skin scrapings	papillomavirus, orf
	molluscum contagiosum

EM staining protocol

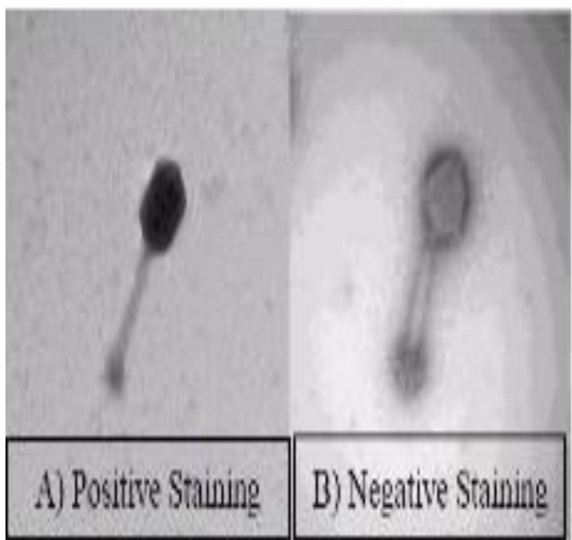
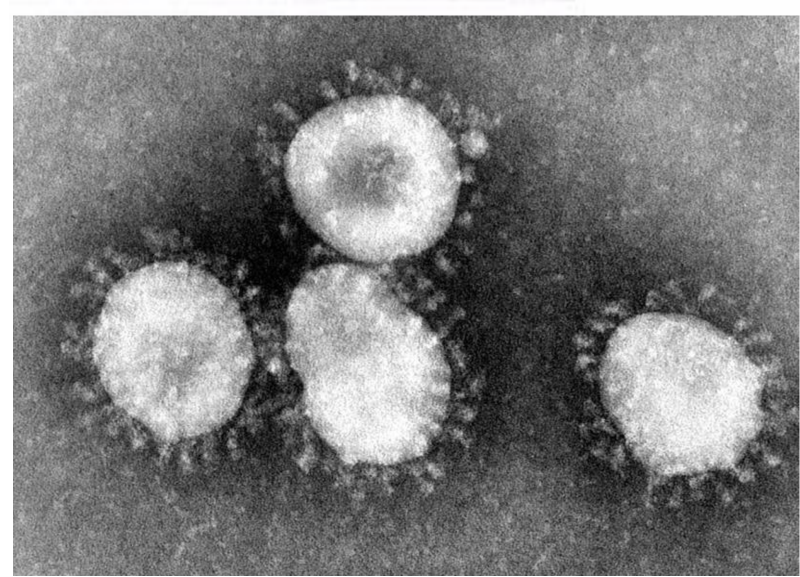


Figure 1. Examples of a positively stained virus (A) and negatively stained virus (B).



Fred Murphy - This media comes from the Centers for Disease Control and Prevention's Public Health Image Library (PHIL), with identification number **#4814**

- **Procedure is light and CO_2 sensitive**
- **In positive staining of virus**, uranyl acetate and/or lead citrate are the most commonly used salts.
- Ultra-thin sections of the samples are incorporated into a copper grid and the uranyl acetate salt added.
- Washing which is a very critical process of the stain is done to eliminate excess salts from outside the stain.
- Airdried grids of the sample are viewed under the **Transmission Electron Microscope**.
- **Advantages**
- It has been used to visualize various morphologies of viruses such as viral spikes, and envelopes, for viruses such as orthomyxoviruses, adenoviruses, hepatitis, rhinoviruses, influenza viruses.
- It is used to study the morphologies and physiological features of viruses.
- It has been used to identify and differentiate various viruses such as animal viruses from human viruses.

Negative Staining of virus:

- If virus particles are coated with stain (positive staining), fine detail may be obscured. **Negative staining** overcomes this problem by staining the background and leaving the virus relatively untouched. The negative stain is moulded round the virus particle, outlining its structure, and is also able to penetrate between small surface projections and to delineate them. If there are cavities within the virus particle that are accessible to the stain, these will be revealed and some of the internal structure of the virus may be disclosed. Osmium tetraoxide, Uranyl acetate
- 2% Uranyl acetate solutions also act as a fixative for **viruses**. The advantage of uranyl acetate and uranyl formate is that they produce the highest electron density and image contrast as well as imparting a fine grain to the image.

Protocol

- EM grid pre-treatment: 300-400 mesh Cu grid. Adding a thin (2-5 nm) carbon layer on top of the plastic film helps to spread efficiently the thermal energy introduced by the electron beam during imaging.
- Particle adsorption: This procedure usually results in higher particle number (ultracentrifuged virus) at the grid surface than putting the grids on a sample droplet.
- Washing: Removal of salts or macromolecules with deionized water, which might interfere with the staining or visibility of particles.
- Incubation with heavy metal stain: Incubation of the adsorbed particles with heavy metal salt solution and drying for embedding them in an amorphous film. Use at least two different stains with different capabilities to reveal different features of the particles (e.g. 1% uranyl acetate and 1% phosphotungstic acid)

Immuno Electron Microscopy

The sensitivity and specificity of EM may be enhanced by immune electron microscopy. There are two variants:-

Classical Immune electron microscopy (IEM) - the sample is treated with specific anti-sera before being put up for EM. Viral particles present will be agglutinated and thus congregate together by the antibody.

Solid phase immune electron microscopy (SPIEM) - the grid is coated with specific anti-sera. Virus particles present in the sample will be absorbed onto the grid by the antibody.

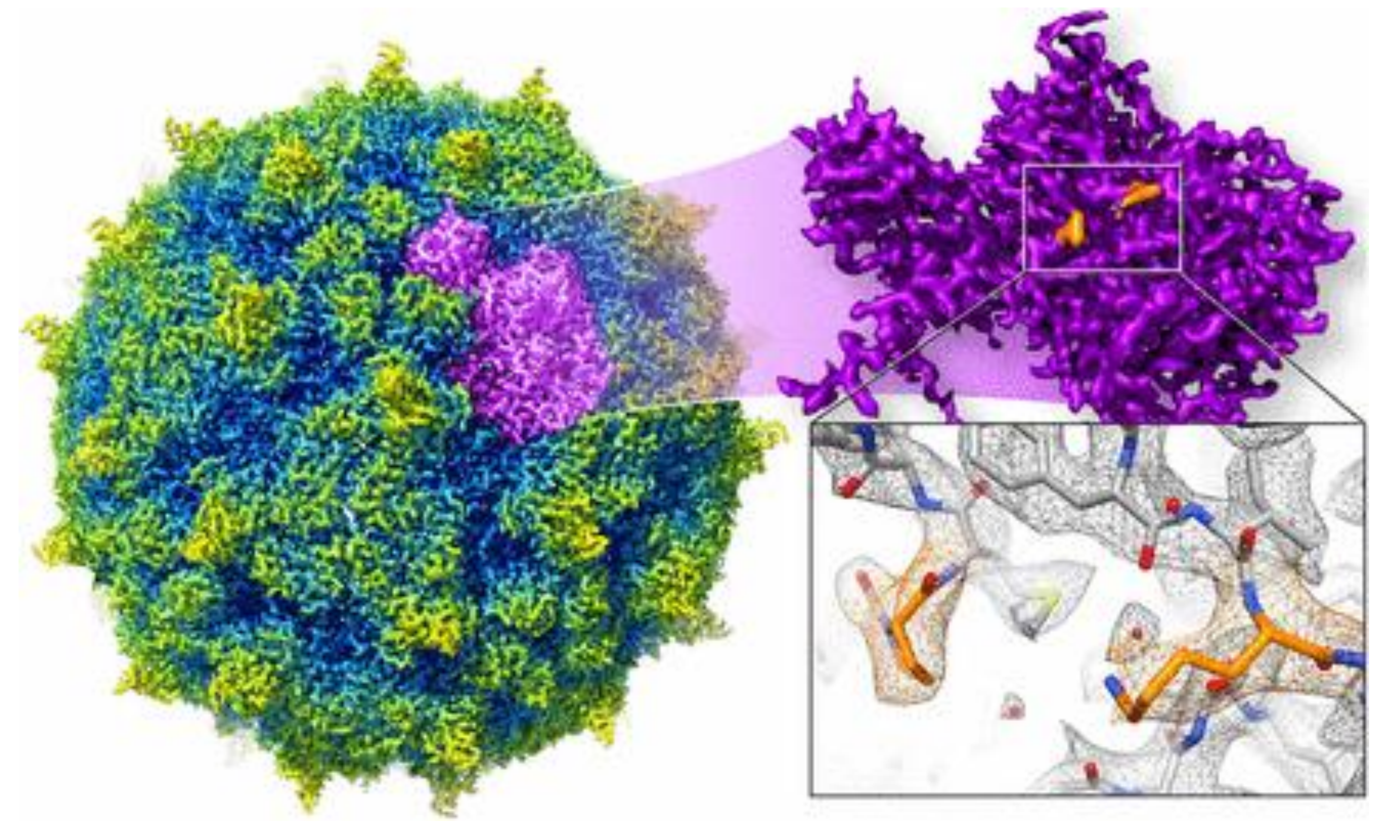
X ray crystallography for virus structure

- Used for last 30 years to determine virus structure
- Require pure virus in large amounts for crystallization process
- The purified virus must also be able to form paracrystalline arrays large enough to cause significant diffraction of the radiation source.
- Useful for large and symmetrical viruses:TMV (helical) and Turnip Yellow Mosaic virus (isosahedral virus) 1st to be studied with XRC
- Enveloped viruses also limitation
- One further limitation is that some of the largest virus particles, such as poxviruses, contain hundreds of different proteins and are at present too complex to be analyzed using these techniques.

Cryo EM and Cryo tomography

- In 2017, the Nobel Prize in Chemistry was awarded to Jacques Dubochet, Joachim Frank, and Richard Henderson "for developing cryo-electron microscopy for the high-resolution structure determination of biomolecules in solution."[4] *Nature Methods* also named cryo-EM as the "Method of the Year" in 2016.
- Samples cooled to cryogenic temperatures and embedded in an environment of vitreous water. An aqueous sample solution is applied to a grid-mesh and plunge-frozen in liquid ethane or a mixture of liquid ethane and propane Visualize multiple high-resolution maps from a single, simply purified, low-concentration sample
- Cryo-EM is ideally suited to exploring the 3D structure of macromolecular assemblies, and elucidation of the 3D arrangement of such complexes helps understand their function in living cells.
- These technological developments have always involved analyses of viruses, particularly plant viruses, because their symmetrical capsids, as well as the availability of highly pure samples, greatly facilitates reconstruction.

https://en.wikipedia.org/wiki/Cryogenic_electron_microscopy



Cryo-EM structure of rhinovirus C 15a, displaying icosahedral symmetry, with spikes (yellow) and valleys in the surface. Each asymmetrical unit (purple) includes a canyon next to the spike; in this canyon, proline and lysine residues (orange) form a pocket likely to interact with sialic acid. Earl et al. PNAS August 9, 2016 113 (32) 8903-8905

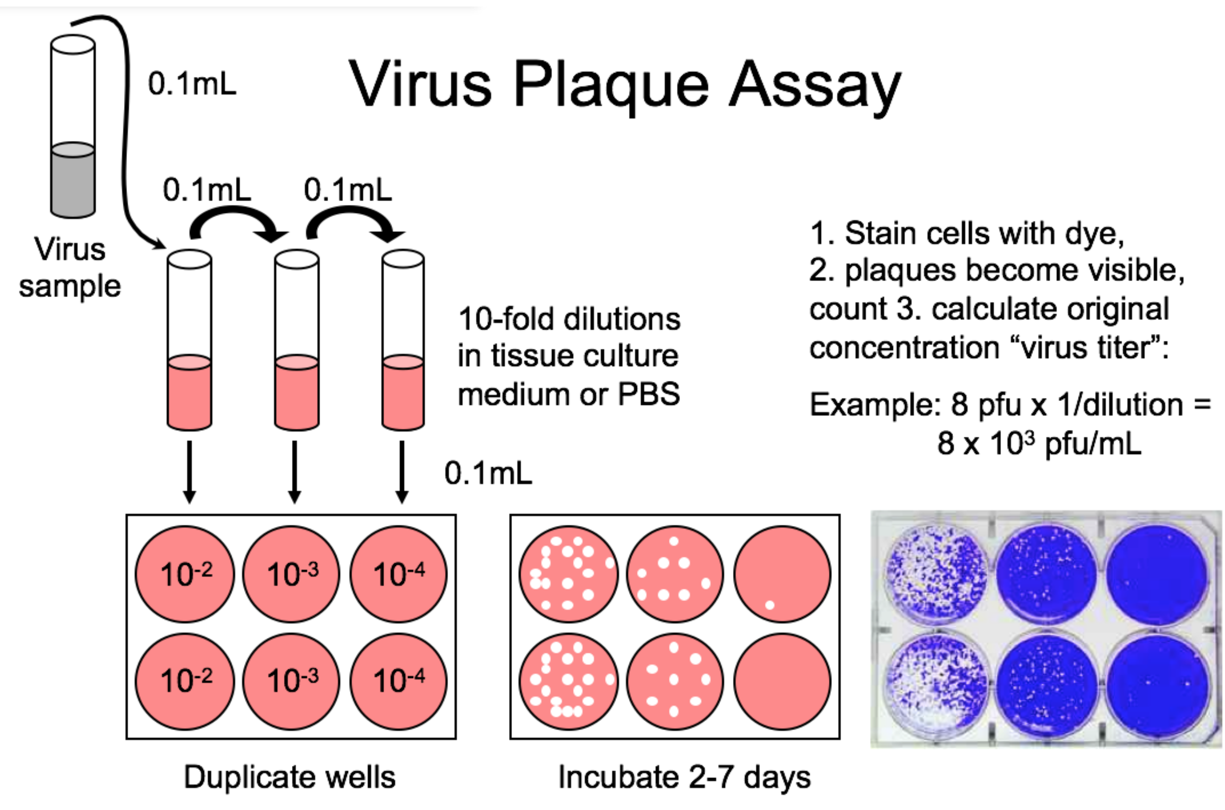
Quantification

- TEM images can show individual virus particles and quantitative image analysis can be used to determine virus concentrations.
- These high resolution images also provide particle morphology information that most other methods cannot.
- Quantitative TEM results will often be greater than results from other assays as all particles, regardless of infectivity, are quantified in the reported virus-like particles per mL (vlp/mL) result. Quantitative TEM generally works well for virus concentrations greater than 10^6 particles/mL.
- Latex beads ratio 10:1 comparison of known and unknown virus samples
- **Disadvantages of Electron Microscopy**
- Expensive equipment, Expensive maintenance
- Require experienced observer,Sensitivity often low

Infectivity Assays

- **Pock Assay: Using Chorio Allantoic Membrane**
- **End Point Assay: Experimental Animals**
- **Plaque Assay**
- When cells grow as monolayers, they can be used to quantify the number of viruses using plaque assay. - The virus is serially diluted in a liquid medium. - For each dilution a set amount is added to separate plate containing monolayer of tissue culture cells and the viruses in that solution are allowed to attach to the tissue culture cells. - After attachment has been allowed to occur, a semi solid medium is added to restrict the movement of new viruses produced so that only adjacent cells will be infected

Plaque Assay



https://memorang.com/flashcards/97240/Week+1+-+Intro+to+Virology

Calculation of pfu

- Serial 10-fold dilution of virus sample are tested(duplicates)
- e.g 10^{-1}-10^{-10} (depending on the virus one is working with
- and the information about the aliquot to estimate the range
- of virus dilution)
- The dilution of virus which gives 20-100 plaques per petri dish
  - is used for calculation of PFU
  - e.g 10^{-5} dilution gives 20 plaques, and 0.5 ml of virus
  - dilution is added to the cell monolayers, then the PFU of
  - the virus stock will be 20 x 10^{5}
  - Divide by 0.5 ml: 4x10^{6} pfu

TCID: Tissue culture infectivity Dose

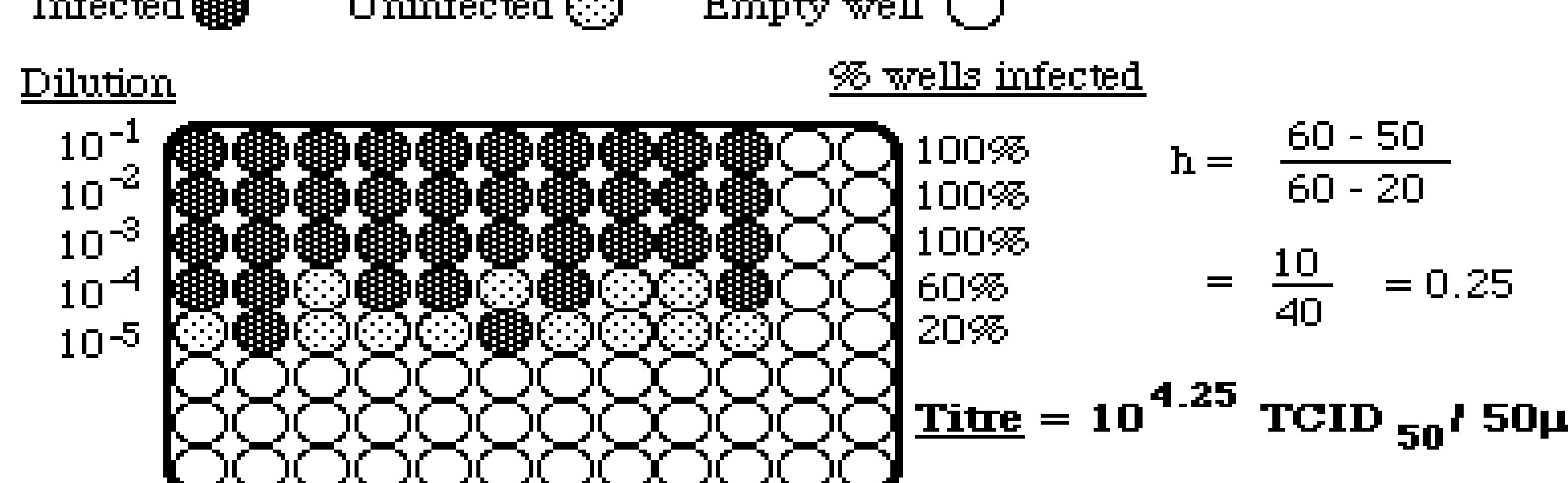
- Mechanism: similar to plaque assay, based on the CPE of virus and measures the infectivity of virus particles
- For viruses which do not form distinct plaque on infected cells
- Use a statistic method for quantification
- Similar procedures as plaque assay, but liquid medium is used for the incubation, no agar is added to the medium, no staining is needed, observe CPE under light microscope
- Minimal 5 duplicate wells for each dilution. Usually 10 well/dilution
- 2 methods used
- Reed and Muench Methods
- Spearman and Karber Method
- 0.69 PFU = 1 $TCID_{50}$ based on the Poisson distribution

Calculation of $TCID_{50}$

- Use of Reed & Muench method (if 50 ul of virus dilution is added to each well)

$$h = \frac{(\%\ \text{wells infected at dilution next above } 50\%) - 50\%}{(\%\ \text{wells infected at dilution next above } 50\%) - (\%\ \text{wells infected at dilution next below } 50\%)}$$

where h = an interpolated $\log_{10}$ value of a dilution step.
This value is added to the step **above** the 50% value.



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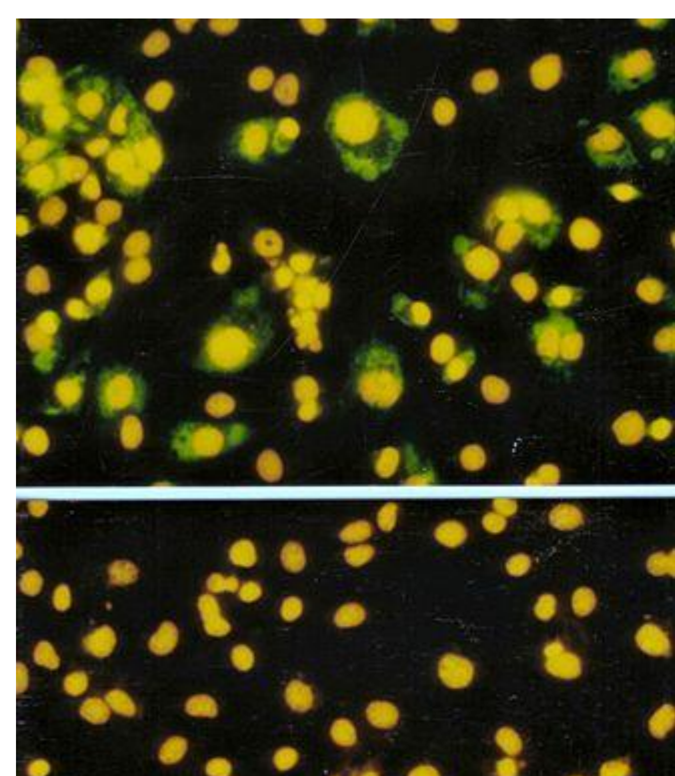
Focus Forming Assay

- The focus forming assay (FFA) is a variation of the plaque assay, but instead of relying on cell lysis in order to detect plaque formation.
- FFA employs immunostaining techniques using fluorescently labeled antibodies specific for a viral antigen to detect infected host cells and infectious virus particles before an actual plaque is formed.
- The FFA is particularly useful for quantifying classes of viruses that do not lyse the cell membranes, as these viruses would not be amenable to the plaque assay.
- Like the plaque assay, host cell monolayers are infected with various dilutions of the virus sample and allowed to incubate for a relatively brief incubation period (e.g., 24–72 hours) under a semisolid overlay medium that restricts the spread of infectious virus, creating localized clusters (foci) of infected cells.
- Plates are subsequently probed with fluorescently labeled antibodies against a viral antigen, and fluorescence microscopy is used to count and quantify the number of foci. The FFA method typically yields results in less time than plaque or fifty-percent-tissue-culture-infective-dose ($TCID_{50}$) assays, but it can be more expensive in terms of required reagents and equipment. Assay completion time is also dependent on the size of area that the user is counting. A larger area will require more time but can provide a more accurate representation of the sample. Results of the FFA are expressed as focus forming units per milliliter, or FFU/mL (https://en.wikipedia.org/wiki/Virus_quantification)



Cells infected by rotavirus-FITC labeled antibody (top) and uninfected cells (bottom)

References

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