



Virology

Doctor 2018 | Medicine | JU

DONE BY

Alia Abbadi

CONTRIBUTED IN THE SCIENTIFIC CORRECTION

Naser Dalqamoni

CONTRIBUTED IN THE GRAMMATICAL CORRECTION

Naser Dalqamoni

DOCTOR

Belal Azab

In this sheet we will be covering the following topics:

- The infectious cycle.
- Virus cultivation.
- Viruses cytopathic effects.
- Viruses quantification plaque assay, end-point dilution assay.

• The infectious cycle

For viruses to grow, there is a specific cycle called the infectious cycle (*sometimes it's called reproductive cycle*). This cycle includes everything that happens from the start (*when a virus attaches to the cell*) to the end (*the infected cell*).

Virologists divide the infectious cycle into stages to facilitate their study, but there are **no** artificial boundaries in this cycle. In other words, the cycle is a continuous process not discontinuous phases.

Just like how the cell has its own cycle of 4 stages (prophase, metaphase, anaphase and telophase) viruses also have their own cycle.

❖ Stages/phases in the virus cycle

STAGE 1: Attachment and entry

This phase starts with the **binding of the virus to a receptor** on the surface of the cell which results in the virus' attachment to it followed by **the entry of the virus** into the cell.

STAGE 2: Translation

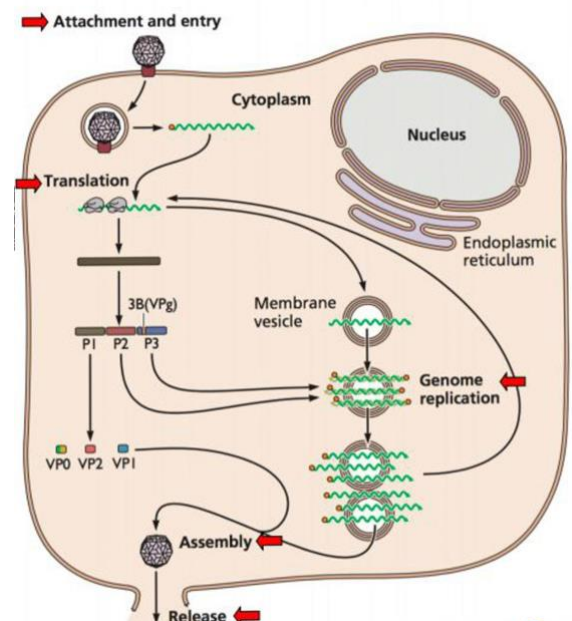
In this stage, the virus **hijacks the translational machinery** of the host cell i.e. the virus will use the cell's RNA polymerase to produce its own RNA + it will use the cell's ribosomes to make its own proteins.

STAGE 3: Genome replication

During this stage the virus will use the host cell's DNA replication machinery to **replicate its own genetic material**

STAGE 4: Assembly and release

Finally, different viral components **assemble together** and after that, the new viruses are **released** outside the cell.



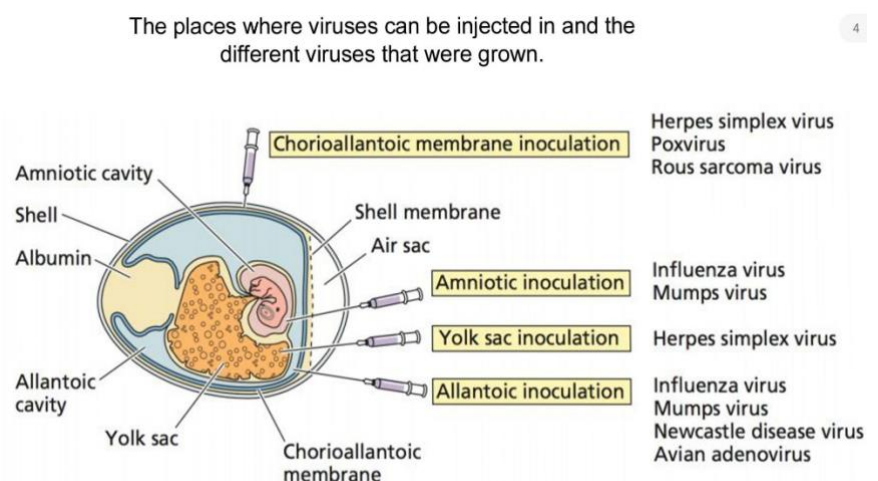
❖ Some important definitions you need to know (terminology)

- A **susceptible** cell has a functional receptor for a given virus to be able to internalize (or at least attach to the cell) - *the cell may or may not be able to support viral replication (it may be able to support it but not necessarily)*
- A **resistant** cell has no receptor – *it may or may not be competent (able) to support viral replication.*
- A **permissive** cell has the capacity to replicate virus- *it may or may not be susceptible [may or may not have a receptor]*
- A **susceptible AND permissive** cell is the only cell that that can take up a virus (has a receptor for it) and replicate it.

❖ Growing viruses is needed for many purposes, but how can we grow them?

One classical way to grow viruses is using **chicken embryos**. Look at the picture below of a cross section of a chicken egg.

- A chicken egg has different components, and in each one a specific type of viruses are inoculated (injected) where they are grown. For example *herpes simplex* virus is inserted in *chorioallantoic* membrane while *influenza virus* is inoculated in the *amniotic cavity*. (The place of inoculation for each virus is NOT for memorization just understand the concept).



The Year 1949

But, cultivation using chicken embryo is *rarely* used nowadays. It was the most popular approach until John Enders, Thomas Weller and Fredrick Robbins were able to grow a virus called poliovirus ***in vitro***; which means they managed to grow it in cells ***outside*** the living system. This takes us to our next topic, *virus cultivation using tissue culture*.



John Enders (1897-1985)



Thomas Weller (1915-)



Frederick Robbins (1916-)

John Enders, Thomas Weller (1915-) and Frederick Robbins (1916-) were able to grow **poliovirus** in vitro using human **tissue culture**. (Nobel Prize, 1954) This development led to the isolation of many new viruses in tissue culture.

- **Virus cultivation using tissue culture**

First let's get a general idea about how a cell culture is prepared:

You **take** the cells → put them in a **petri dish or a flask** → add **media** to these cells [media consists of sterile water + ingredients important for the cells like carbs (sugars) and proteins] → resultant cells adhere to the surface of the flask/ dish.

Nowadays, we have many different kinds of cell cultures which we use to grow viruses in. We'll be talking about 3 of those which are: primary cell lines, immortal cell lines and diploid cell strains.

1) Primary Cell lines

You **take** a tissue from a human or (an animal) → **chop it up** with tweezers → **digest** it using trypsin → Why? because trypsin partially dissolves the **extracellular matrix** thus separating cells from each other and giving us individual cells. → After that, **culture** the cells in a flask/dish.

❖ Cell culture dishes /flasks/plates come in different sizes

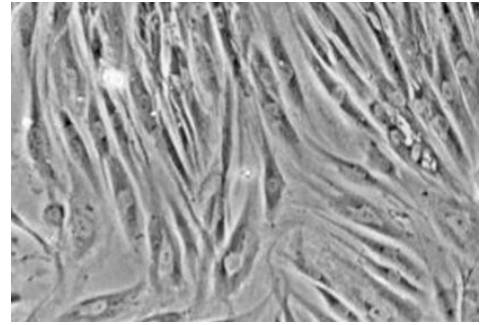


After culturing the cells, you put them in an **incubator** (as shown in the picture) which does two important things:

- A) It keeps the cultures **sterile** because it has filters.
- B) It mimics the **physiological conditions** in our bodies: e.g. maintaining a temperature of 37° C and a CO₂ level of 5%.



- ❖ Most famous example on primary cell lines is **foreskin fibroblast** which is removed from the tip of a baby's penis during circumcision.



Interesting information: It turns out that we can culture foreskin cells to obtain **fibroblasts** from which collagen inserted into cosmetics can be made. Also, these fibroblasts release **fibroblast growth factor "FGF"** which can be injected in the face to keep it healthy.



Child Strapped down



Foreskin surgically removed from penis, no anesthetic used.



Fibroblasts from the cells of the foreskin is used to make collagen



Foreskin collagen inserted into womens skin care products

- ✓ Now check the following table of the advantage and disadvantage of PCL

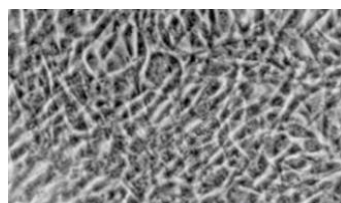
Advantage of primary cell lines	Disadvantage of primary cell lines
They mimic actual cells inside our bodies since they are taken straight from there.	They are mortal i.e. they don't live forever. Their division capacity is just 20-30 times, after that they die. That's because cells age as a result of telomeres shortening. (This is also the case in our bodies)

2) Immortal cell lines

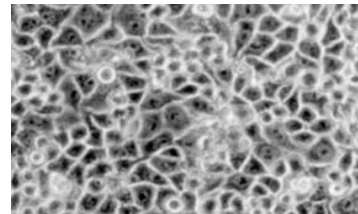
These cell lines live forever and *never die*, hence the name "**immortal**".

- Examples of ICL:

Mouse fibroblast cell line (3T3) and **human epithelial cell line called (HeLa)**.



Mouse fibroblast cell line (3T3)



Human epithelial cell line (HeLa)

*HeLa: Abbreviation of the first 2 letters of the first and last name of a patient with cervical cancer who died in the 50's. During surgery, cells were taken from that tumor and cultured.

- HeLa cell lines can be frozen in liquid nitrogen and be used whenever needed since they never die.
- The following table shows the advantage and disadvantage of immortal cell lines.

Advantage of immortal cell lines	Disadvantage
Convenient and easy to use, we don't have to get them from donors. Plus, they live forever.	Just like cancer cells, the genetic material is messed up. For example, the number of chromosomes might not be 46. And even if it was 46, there would be abnormalities, deletions, duplications, mutations in the genetic material. Thus, these cells don't resemble normal healthy cells.

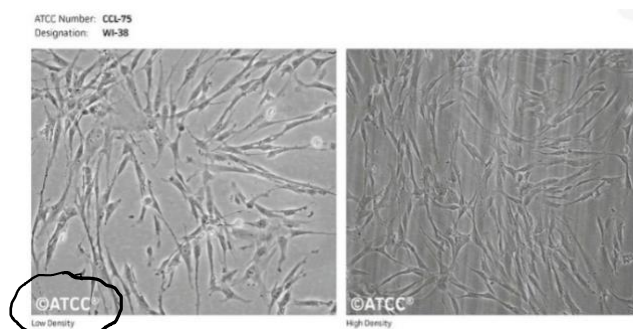
➤ Now, the question is: what type of cell lines do I choose?

If you desire to grow **a large number** of viruses, **immortal cell lines** are a better option because genetic material of the cells doesn't matter here. Whereas **mortal cell lines** would be preferred if you want to study **the effect of the virus on the cell**, since we need cells that resemble normal ones.

3) Diploid cell strains

These cells have the following characteristics:

- ✓ They have the **right** number of chromosomes (46)
- ✓ They are **not immortal** but they last *longer* than primary cell lines



One example is: **WI 38 cells**.

***ATCC**: is an organization/bank from which you can buy cell lines and get a lot of information about them (e.g. if individual was normal/diseased, type of disease..etc)

Before we start get to the next point, please watch this short video:

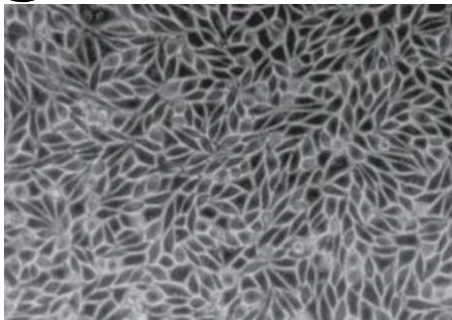
<https://youtu.be/2xzHSMuLqII>

The video you just watched was of cells **dividing and filling** the spaces between them. It then gets to the point where they become **confluent**, which means that the cells have covered the entire area and no spaces are left between them. After that there are two possibilities: if they are **normal** cells → they would **stop** dividing when they become confluent but if they are **cancer** cells → they would **continue** dividing even though contact inhibition between neighboring cells was supposed to stop the division.

- **Viruses cytopathic effects (CPE)**

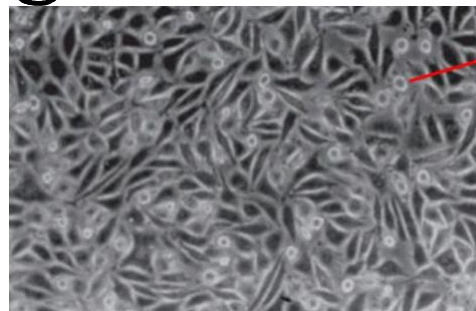
When we add the virus to a cell line how do we know if the virus is growing in these cultured cells? Well, when cells get infected by viruses they undergo several changes known as **virus cytopathic effects**. Since these effects can be seen on microscope, here are pictures showing a series of panels of cells infected with poliovirus. After that, watch the following video of cytopathic effect of *Herpes simplex virus 1* infected cells: <https://youtu.be/lwRX53Y6pAg>

①



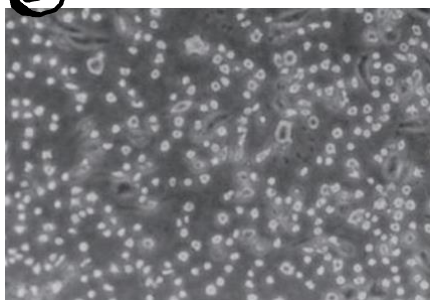
A monolayer of **confluent** cells adjacent to each other (before the viral infection)

②



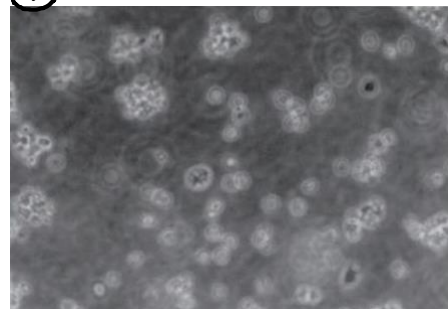
After 2 hours of the infection few cells are becoming **round** and starting to **detach** from other cells

③



After even more time, most of the cells have **rounded up** and are **floating around**

④

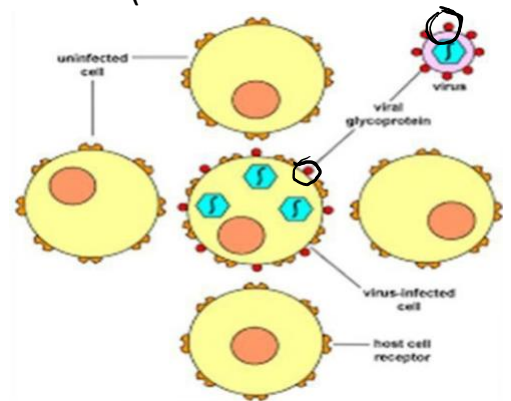


At the end, infected cells are all **rounded up, detached** from each other and the surface of the flask, they become **dead cells floating** in the medium

Rounding up, detachment and dying is one kind of a cytopathic effect but there are actually different types of CPE depending on the cell and the virus.

❖ A second type of CPE is formation of **syncytia**. A syncytium forms when cells fuse together. To explain how it occurs let's take the HIV infection which occurs mostly in the brain as an example:

- 1- First, the virus **binds to specific receptors** on host cell due to the presence of a glycoprotein called **gp120** on the virus' surface (this allows it to internalize).
- 2- The virus starts **expressing its own components** using the cell's machinery, in this case, including the gp120. As a result, the host cell will have gp120 -which is a viral protein- on its membrane.
- 3- The viral protein can **bind to receptors on neighboring cells**, and this will cause uninfected cells to bind to the infected one causing them to **fuse** together.
- 4- Formation of the **syncytium** which is highly unstable leads to **cell death**. In the case of HIV, it causes brain death.

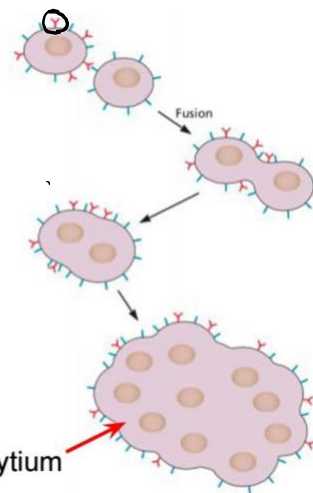


You probably noticed the important role of gp120 in the formation of the syncytium, this protein is what the virus uses to get in cells to reproduce. And when expressed on the infected cell's surface, this viral protein catalyzes fusion of cells together. Therefore this cytopathic effect is a characteristic of viruses that encode fusion proteins.

A light microscopy image of a syncytium



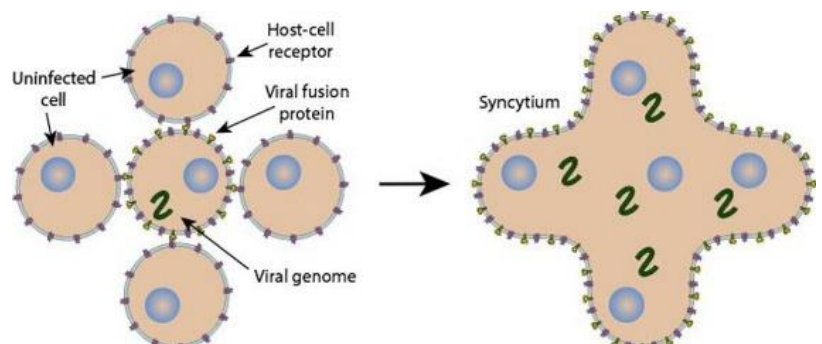
Syncytium



The Y-shaped red membrane protein is the viral protein

Large abnormal multi-nucleated cell (syncytium)

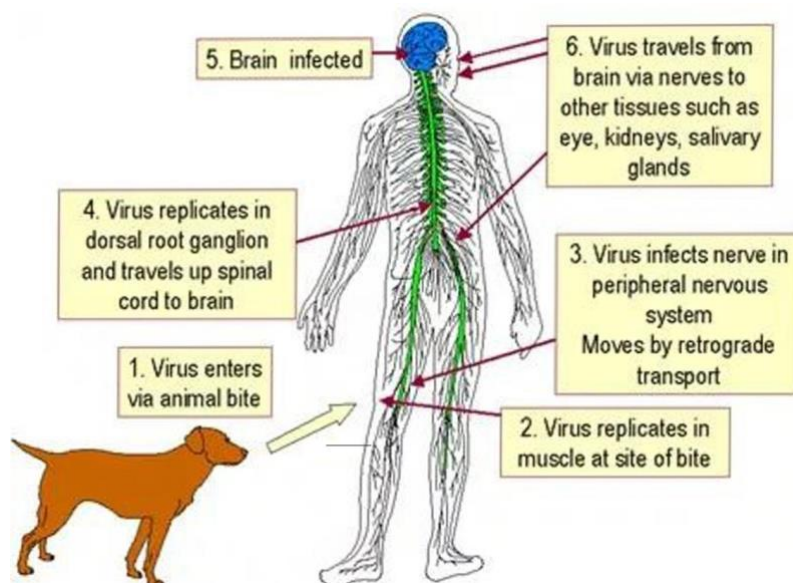
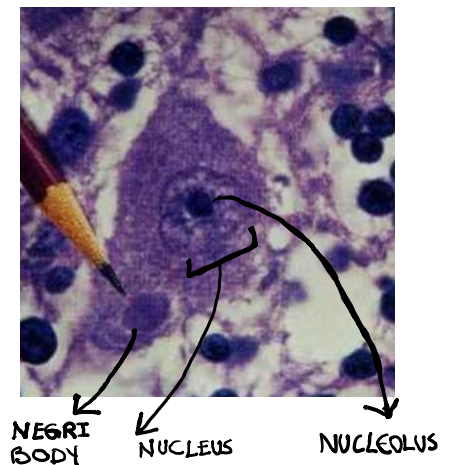
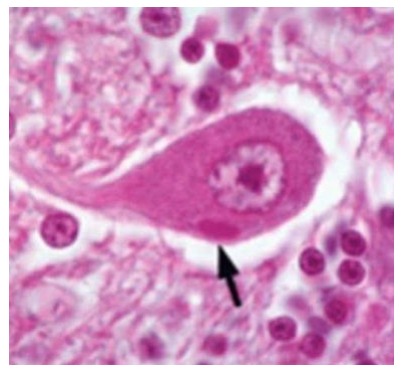
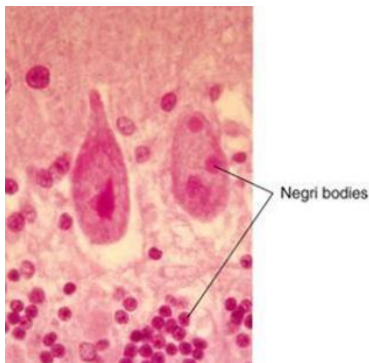
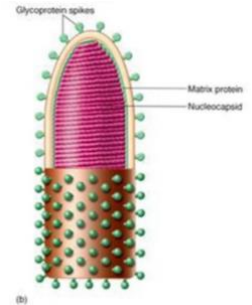
Question: why does the cell even have receptor for the virus? The receptor on the host cell is actually there for **other important functions** but the virus uses it for its own benefit. E.g. HIV infects white blood cells and internalizes after binding to specific receptors on these cells which have a role in immune response.



- ❖ Some of CPE can be a **diagnostic** sign for the virus. For example; **Negri bodies** - which are a type of **inclusion bodies**- are looked for in diagnosing Rabies virus infection. This is the last CPE we'll be talking about.

Rabies virus:

- ✓ Its genus is *Lyssavirus*.
- ✓ Its genetic material is **ssRNA** enveloped by a nucleocapsid.
- ✓ It has a shape of a **spiked bullet**.
- ✓ It multiplies in the brain forming bodies called Negri Bodies which are: **eosinophilic, sharply outlined, pathognomonic inclusion bodies found in the cytoplasm of certain rabies infected nerve cells.**
- ✓ Humans usually get infected by rabies virus from *pet bites*, these pets also usually get the virus from *wild animal bites* like a raccoon or a bat, that's why pets should be vaccinated against rabies virus.



- ❖ This table shows more examples on viral cytopathic effect you're only required to know about the ones we've already covered.

Cytopathic effect(s)	Virus(es)
Morphological alterations	
Nuclear shrinking (pyknosis), proliferation of membrane	Picornaviruses
Proliferation of nuclear membrane	Alphaviruses, herpesviruses
Vacuoles in cytoplasm	Polyomaviruses, papillomaviruses
Syncytium formation (cell fusion)	Paramyxoviruses, coronaviruses
Margination and breaking of chromosomes	Herpesviruses
Rounding up and detachment of cultured cells	Herpesviruses, rhabdoviruses, adenoviruses, picornaviruses
Inclusion bodies	
Virions in nucleus	Adenoviruses
Virions in cytoplasm (Negri bodies)	Rabies virus
"Factories" in cytoplasm (Guarnieri bodies)	Poxviruses
Clumps of ribosomes in virions	Arenaviruses
Clumps of chromatin in nucleus	Herpesviruses

After studying about virus infectious cycle, virus cultivation and virus cytopathic effect, now we'll talk about *virus quantification*, which is important for knowing **how many viruses the infected cell is producing**.

- ❖ Virus quantification can be divided into 2 broad categories:

1- Measuring the **physical particles** of the viruses, their parts and components. (how many virus particles in a certain sample) e.g. a sample contains 2 million viral particles "VP"/ml.

⇒ But, *not all viruses are actually infectious* and sometimes we need to quantify these, which is why we have the second approach.

2- We can measure **infectivity** (how many infectious viruses (called virions) are in a sample per ml)

- ❖ The most commonly used methods to quantify viruses can be subdivided into:

- Techniques measuring the **viral infectivity "virions"**.
 - a) **Viral Plaque Assay** (the gold standard method)
 - b) **TCID50**
- Techniques that examine **viral nucleic acid and protein** (we can quantify viruses based on their genetic material or proteins)
 - a) **qPCR** (quantitative Polymerase Chain Reaction) or real time PCR

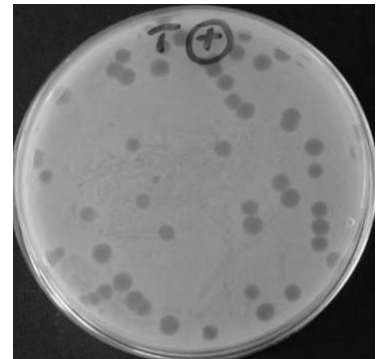
Remember that PCR is used to amplify a specific region of DNA (*or RNA, but in this case a reverse transcriptase is needed first to generate DNA molecules from RNA*). If I add primers, unique to a certain region of the virus' genetic material and amplify it by PCR, the end products' quantity will give me an idea

about the initial quantity of the viral genetic material, and that's because amplification is *exponential*. [We will talk about quantitative PCR (qPCR) more in the next lectures]

- b) **Western Blotting**: Allows us to look at viral protein and from the amount of that protein we can know how many viruses are present in a sample. [will also talk about it later]
- **Immunoassays (ELISA)**: using specific *antibodies* to detect the virus.
- Techniques that rely on **direct counting** of physical viral particles
 - a) **Transmission Electron Microscope**. (actual counting of viral particles under electron microscope)
 - b) **Viral Flow Cytometry**: cells, bacteria or viruses pass through very narrow needle determining how many particles are there.

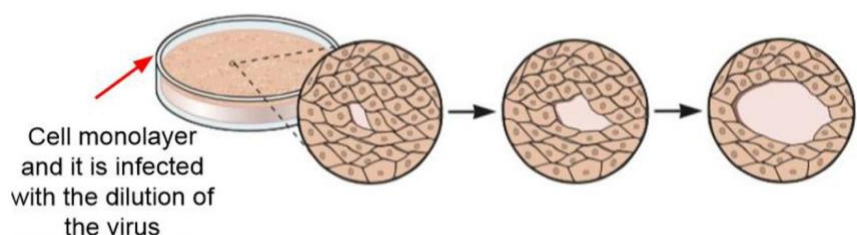
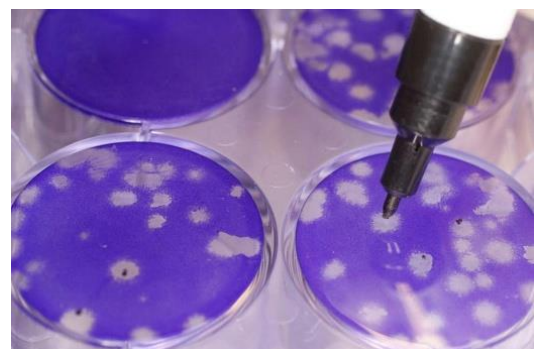
Now, let's talk more about the gold standard method for quantifying (titering) the number of ***infectious particles*** in a sample, this method is the **plaque assay**:

✓ Was first developed in 1930s for people who were studying *bacteriophages*, they noticed how it becomes cloudy in the dish where the bacteria is growing but upon adding the virus a *clear zone* appears where the virus is killing the bacteria → Each clear zone arises from one viral infectious particle → The number of plaques gives an estimation about the number of the viruses in the sample → It is called **plaques forming units per milliliter (PFU/ml)**



✓ Renato Dulbecco developed plaque assay for *animal viruses* after about 20 years. He received a noble prize in 1975 for a number of discoveries including this one.

This method involves the following steps: Grow a **monolayer of cells** in a petri dish → add an **overlay of agar** → add the **virus** → cells become infected producing viruses that **infect even more cells** → **clear areas (or plaques)** appear on the monolayer where the cells died upon ***viral infection***.



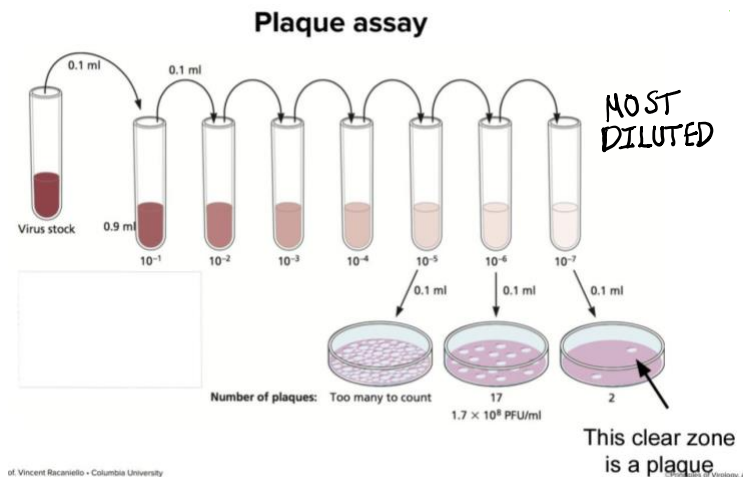
❖ How do I quantify virions using plaque assay?

First you need to know that **1 plaque originates from 1 virion** so knowing the number of plaques would help knowing how many virions there are in the sample. One problem is that the number of virions in the original sample might be *large* producing a lot of plaques which would be *hard to count* so we do **ten fold serial dilutions** as follows:

1- **0.1ml** of the original sample is mixed with **0.9ml of water**, then another 0.1ml from the second sample is taken and mixed with 0.9ml of water, then 0.1ml of the third sample is mixed with 0.9ml of water. At this point, **the dilution factor** is 1/1000 or 10^{-3} .

2- Dilutions will be **repeated** until let's say, a dilution factor of 10^{-7} .

3- Then **we take some liquid** from the samples. If we *have an idea* about what the titer (quantity) is going to be, we take from specific samples, *if not*, we take from **all** samples. For example, here we just take from the samples with dilution factors of 10^{-5} , 10^{-6} and 10^{-7} some liquid and put it into *three different cell cultures*, which we know are **susceptible and permissive** to the virus, and then we cover these plates with **agar** overlay and **incubate** them overnight.



4- **Plaques** will be formed, then we **stain** the monolayer to see the plaques very clearly. We **count** the plaques appearing on each plate:

- ✓ 10^{-5} plate is rich in **overlapping plaques** that we can't count (not convenient).
- ✓ 10^{-7} plate has **only two plaques** which may not be statistically significant (not convenient).

⇒ So, we have to find a plate that has enough number of plaque which we can count → the 10^{-6} plate in this example (17 plaques).

5- We then use the following **formula** to calculate the original concentration of the plaques & thus virions:

$$\text{Virus titre} = \frac{(\text{Number of virus plaques}) \times (\text{Dilution factor})}{\text{Volume of applied virus solution (mL)}} \quad (\text{To the plate})$$

In this case → $17 \times 10^6 / 0.1 \text{ ml} = 1.7 \times 10^8 \text{ PFU/ml}$

The thing is, we actually do plaque assay for the chosen sample with the **same** dilution factor **twice** and take the **average** of the two concentrations to be more precise because we usually don't get the same number of plaques every time. EXAMPLE:

- E.g. After applying 200 μL of a virus 10^4 dilution, 40 plaques in well A, 45 plaques in well B. What is the pfu/ml?

APPLY THE
PREVIOUS
FORMULA IN
EACH CASE

$$\text{Virus titre in well A} = \frac{40 * 10^4}{0.2 \text{ (mL)}} = 2 * 10^6 \frac{\text{pfu}}{\text{mL}}$$

$$\text{Virus titre in well B} = \frac{45 * 10^4}{0.2 \text{ (mL)}} = 2.25 * 10^6 \frac{\text{pfu}}{\text{mL}}$$

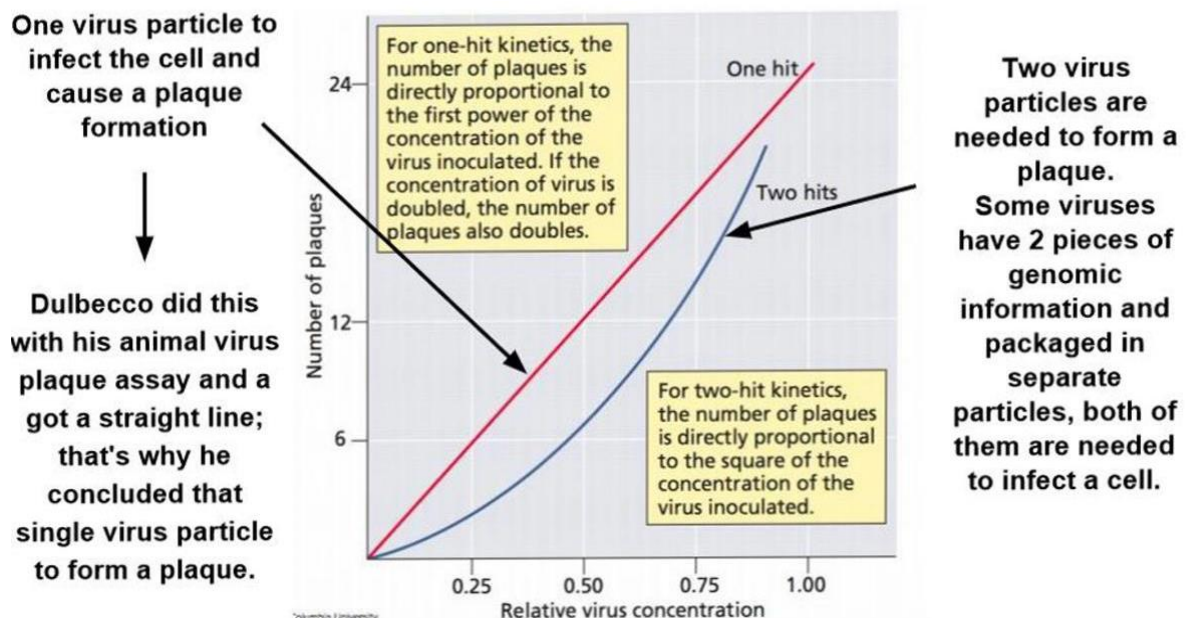
$$\text{Average virus titre} = \frac{(2 + 2.25) * 10^6}{2 \text{ (mL)}} = 2.125 * 10^6 \frac{\text{pfu}}{\text{mL}} \approx 2 * 10^6$$

Watch the following video of how a plaque forms <https://youtu.be/zcmwEkoFWkl>

Remember that there is a **solid agar overlay** on top of the monolayer which is restricting the diffusion of the virus, that's why what we get is **discrete** plaques. If the overlay is *liquid*, the virus will spread throughout the cells which will be dead in a day or two, and there will be no plaques to count.

❖ Dose response curve.

To draw a dose response curve: you make dilutions of a sample and then observe what happens. This can be done with drugs or with viruses.



- It is an XY plot.

- The **X-axis** is the *relative virus concentration* and it is increasing from left to right, these values are dilutions plotted in the opposite way. (A *relative virus concentration of 1.00 means that its not diluted at all 100% of virus found*)
- The **Y-axis** is *the number of plaques*.
- In the case of viruses, do a series of dilutions for the wanted virus, plaque it out and count the number of plaques. Then correlate the relative virus concentration with the number of plaques formed
- Whether you get lines or curves, it indicates if one or more viruses are needed to form a plaque.

Here, we have 2 types:

- ✓ **Two hits curve (the blue one)** indicates that *two viral particles together* are needed to form a plaque, each alone won't be able to do so, this happens in plant viruses.
- ✓ **One hit curve (the red linear one)** indicates that *one* infectious virus is needed to infect the cell and form a plaque. Based on this curve: if we have an absolute sample of virus stock (**no dilution**), then the relative virus concentration will be 1. Thus, we can know how many plaques will be formed. We can also predict the number of plaques when the sample is diluted, so if we look at the point where relative concentration is 0.50 (50% diluted) we can predict the amount of plaques formed.

❖ Application on virus titering

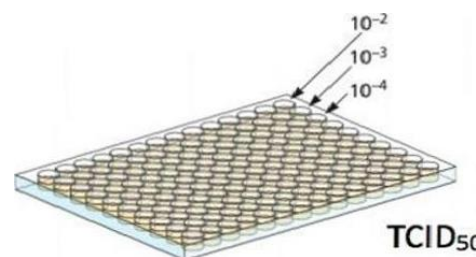
Nowadays, scientists are studying gene therapy using viruses especially the AAV (*adeno-associated virus*). And to do so, quantification of the virus is important. They could use **PCR (Polymerase Chain Reaction)**, in which amplifying the genetic material helps in determining the number of viral particles/ml. However, the important quantification needed is the *infectious quantification* (number of PFUs/ml) i.e. *How many infectious particles enter the cells, infect them, deliver the therapeutic gene and express it.*

● End-point dilution assay

Sometimes, we can't use the PFU method because some viruses **don't form plaques** and **some are hard to work with** when it comes to the agar overlay (e.g. *the virus cannot infect the cells when agar is present*). In these complications, the solution would be to culture the viruses in *liquid media*, which brings us to another type of assay that would still allow us to quantify infectivity

❖ End-point dilution assay is typically done in **96-well plates**.

Host cells are put in each well with *an appropriate medium and serial dilutions* (10^{-2} to 10^{-7} for example) of a virus stock are prepared → Wells are then *infected with diluted samples*. Let's say, each 10 wells, for example, are inoculated with **the same dilution** of virus sample → Then the cultures are *incubated* → *Observation* of the cells to know which ones show CPE.



Each horizontal line is the results of 10 cell cultures for the same dilution

Virus dilution	Cytopathic effect									
10^{-2}	+	+	+	+	+	+	+	+	+	+
10^{-3}	+	+	+	+	+	+	+	+	+	+
10^{-4}	+	+	+	+	+	+	+	+	+	+
10^{-5}	+	+	+	+	+	+	+	+	+	+
10^{-6}	+	+	+	+	+	+	+	+	+	+
10^{-7}	+	+	+	+	+	+	+	+	+	+

“+” means that the cell culture showed CPE, we count those for each dilution.

- At low dilutions (10^{-2} and 10^{-3}) every culture is infected (10 wells showed CPEs out of the 10 inoculated) → 100%
- At 10^{-4} dilution: 9 wells are infected, 1 is not.
- At 10^{-5} dilution: 5 wells are infected, 5 wells aren't.
- At 10^{-6} dilution: just 1 well is infected, 9 wells aren't.
- At higher dilution (10^{-7}), none of the cell cultures are infected; because no infectious particles are delivered to the cells.

➤ Question: for the same dilution, why do some wells show CPEs, while others don't?

Even though samples are taken from the same tube of the same dilution, just some of the wells get virions and that's because distribution is **randomized**.

❖ **The end-point** is the dilution of virus that affects **50%** of the test units (50% CPEs). In this example, the end-point is at the dilution (10^{-5}), where half of the wells displayed CPEs and half of them did not. This number is called **infectious dose 50% (ID50)**.

❖ Most of the time (ID50) comes between two dilutions and is calculated using math.

➤ Question: If the ID50 of a certain virus for a patient is 10^{-5} and the ID50 of the same virus but for a different patient is 10^{-7} which patient has more viruses?

The second one, since he/she has an ID50 of 10^{-7} which is 100 times more diluted than the 1st patient. (It took more dilutions to reach ID50 which means that originally, the concentration of virions was higher)