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Microbiology

Doctor 2017 | Medicine | JU

Sheet Slides

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Topics to be discussed in this sheet:

- Particles-to-PFU
- Single-step and multi-step growth cycles
- Multiplicity of infection (MOI)
- Physical measurements of virus particles
  -Hemagglutination
  -Electron Microscopy
  -Viral enzymes: measuring the viral activity through measuring the viral enzymes.
  -Serology
  -Nucleic acid: PCR, real-time PCR, deep sequencing

Particle to PFU Ratio:

Not all viral particles are infectious, this ratio shows the total number of particles compared to the total number of infectious particles.

(number of physical particles)/ (number of infectious particles i.e. plaque forming units) = Particle: PFU

★One way of counting the number of physical particles -> look at them under the electron microscope

★ Counting the number of virions: 1) Plaque Assay (PFU), 2) End point Assay

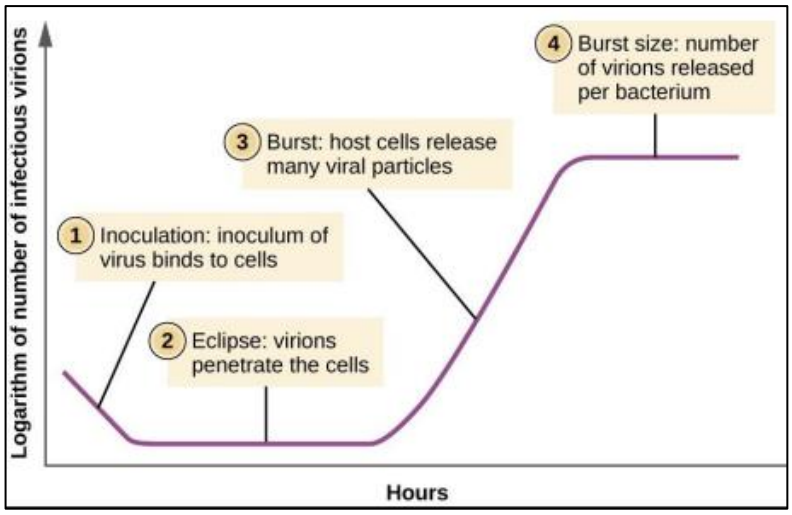
Most viruses do NOT have a 1:1 ratio, and this can be due to damage to the viruses, mutations that cause infectivity to decrease, a complex infectious cycle, or simply that the virus doesn't follow one-hit kinetics (remember, one-hit kinetics means a single viral particle can cause infection).

Virus	Particle-to-PFU ratio
Adenoviridae	20–100
Alphaviridae	
Semliki Forest virus	1–2
Herpesviridae	
Herpes simplex virus	50–200
Orthomyxoviridae	
Influenza virus	20–50
Papillomaviridae	
Papillomavirus	10,000
Picornaviridae	
Poliovirus	30–1,000
Polyomaviridae	
Polyomavirus	38–50
Simian virus 40	100–200
Poxviridae	1–100
Reoviridae	
Reovirus	10

➔ Looking at this table, we can see that the particle to PFU ratios can have a wide range, but they are very easy to read. Looking at the poliovirus, we see that the particle to PFU ratio is 30-1,000 which means there is one infectious particle for every 30-1,000 particles.

Viral Growth Cycles:

When a virus is growing in a cell population, it follows a special type of curve (depending on how much of the virus was initially added to the culture). There multi-step curves and single step curves.



The graph shows the number of free viral particles in a plate at a time, which means the viruses that are bound to cells, or have already penetrated the cells will not be visible when counting. This is the cause of the **initial drop** in viruses at the beginning of the graph. The important parts of the graph are the *eclipse* and *burst* phases.

◇Quick Recap on Growth Curve:

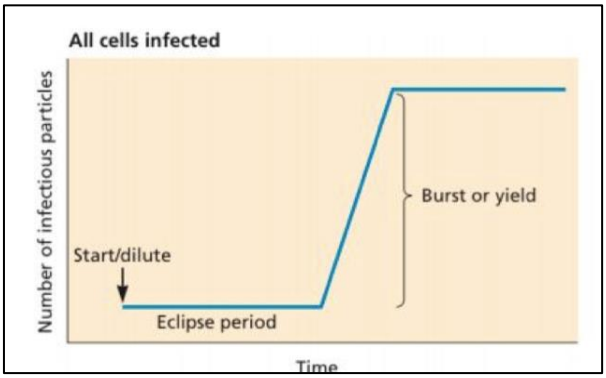
Inoculation: Viruses are added to cells. The drop is due to the reason mentioned previously.

✦*Eclipse phase:* number of infectious particles is not changing because-> virions have penetrated into the cells, their DNA is being replicated, their viral proteins are being synthesized, and finally their components assemble and are ready in order to leave the host cell. (Apparently no infectivity is being generated, but lots of synthesis in the cell is going on)

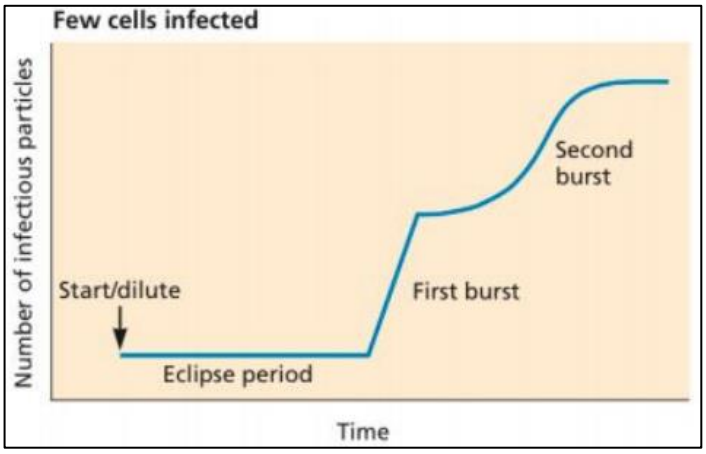
✦*Burst phase/yield phase:* this happens when the first viruses generated leave the cell.

The plateau: the cells will die and the viruses eventually exit them.

-> In the example mentioned above (single step cycle), the only way for the virus to be single step, is for **all of the host cells to be infected simultaneously**. This is so that the eclipse and burst periods will be synchronized where the host cells are all going through the same phase of the replicative cycle and they release all the viruses at the same time.

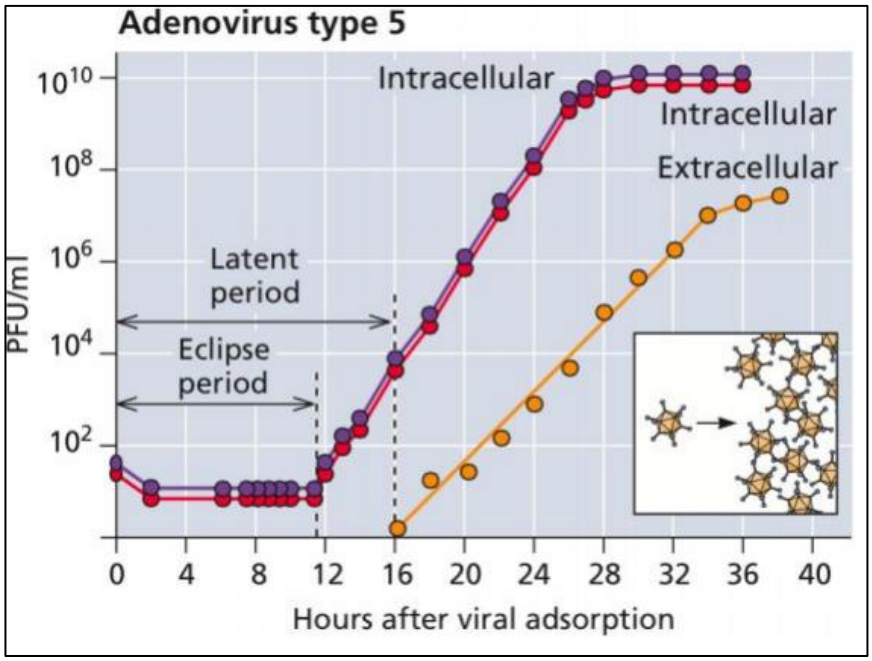


The next example will model what happens if only a fraction of the host cells gets infected at the inoculation period. This is known as the Multi-step growth cycle.



->In this case, a small percent of the host cells is infected at the inoculation. These infected cells start producing the virus and end up bursting. During the first burst phase the healthier uninfected neighboring cells get infected, then they start synthesizing more viruses, and finally burst, causing the second burst phase.

In other words, one population is infected and will burst. The product of that burst will infect the second population.



◊Another important concept that is shown on some graphs is the **number of extracellular viruses in a sample**. The period of time it takes for extracellular viruses to be found in the medium is known as the latent period.

-More elaboration: The eclipse period (where no infectious viruses are in the infected cells-> they are still being formed) took 12 hours. The yield phase started after. The extracellular viruses took 16 hours to appear (Yellow Curve). This is called the latent period. Also known as the period where there was no infectivity outside the cells. There is a gap between the eclipse period and the latent period -> a lag. The reason for this lag is the fact that you are waiting for the viral particles to assemble together and leave the cells out into the medium.

Bacteria VS Viruses: When a bacterium is put in a broth it begins to divide almost immediately, and the growth will be logarithmic because bacteria divide by binary fission. Viruses do not do that, they have to go into the cells to express their genetic

information, and make the parts to build the virus particles. That takes time (the eclipse period/ plateau) and its **length varies depending on the virus.**

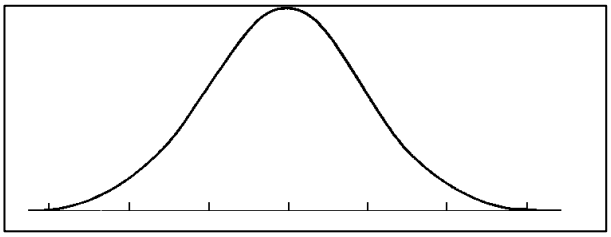
*Now, if we recall, the key or requirement for single step growth is ***the synchronization of infection for the entire population***. All cells need to be infected at the same time for a sample to follow single step curves. Question: How is this done?

> When preparing a sample of cells, another ratio is calculated called the ***multiplicity of infection (MOI***). It is the ratio of **infectious particles** ADDED **per cell.** For example, if we are adding ten million infectious particles to a sample with one million cells, the MOI will be 10. Despite that, we know that not all cells will receive 10 infectious particles. (Keep in mind: There is a difference between adding viruses to the cells and the cells receiving the virus.)

Infection depends on the random collision of virions and cells. When susceptible cells are mixed with viruses, some cells won't be infected, others receive one, two, three or more particles.

⬣ The number of infectious particles each cell receives follows a ***Poisson Distribution***, meaning that the majority of cells will receive roughly the same number of particles, while few cells will receive more or less than average. The shape of the Poisson distribution follows an equation: (It is a normal distribution curve)

$$P(k) = (e^{-m}m^{k})/k!$$



Where:

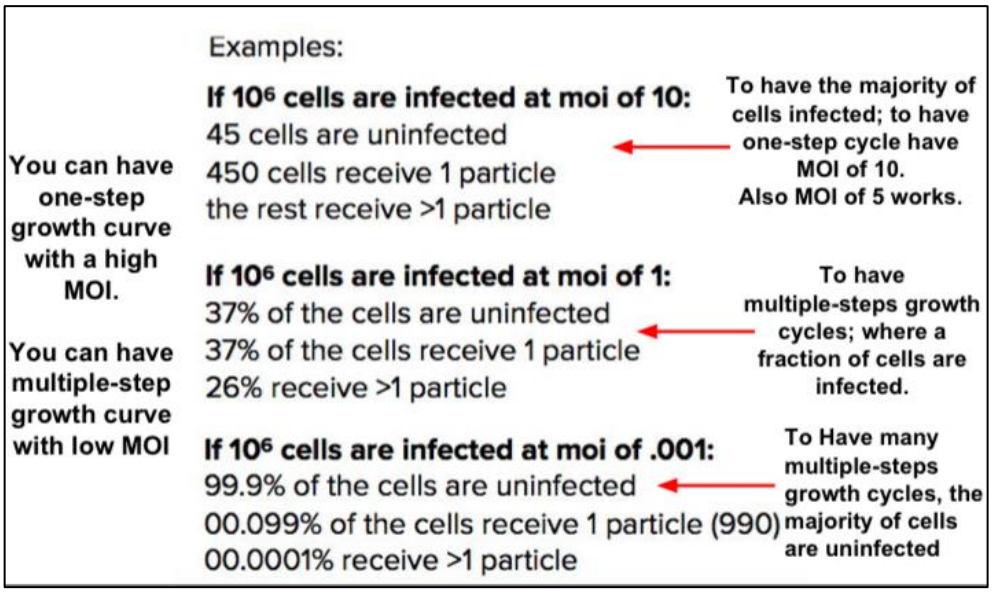
- P(k) = fraction of cells infected by k virus particles (cells receiving the virus)
- K = number of viruses received
- e= natural algorithm
- m = MOI

-In order to find the number of uninfected cells, we use 0 for k, which gives us $P(0) = e^{-m}$

-To find the number of cells that received 1 virus we use 1 for k; $P(1) = me^{-m}$

-To find the number of cells that received >1 virus, we simple subtract the sum of the fraction of the uninfected cells and cells that received 1 virus from the total fraction of cells (the total for all values of p(k) will always be 1 because in the end it is a fraction from a hundred) $P(>1) = 1 - e^{-m}(m+1)$.

-> In the first example shown below, a small percentage of the cells is uninfected (45 cells) which means most of the cells are infected (with a high MOI) and thus allow a one-step growth cycle -> they will all be infected simultaneously. (A MOI of 2 digits -> 10 or more demonstrates a one-step growth cycle/ a MOI of 1 or less -> multi-step growth cycle)



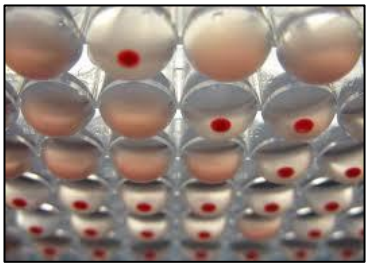
The main take away from this, is that with a high enough MOI, we are able to have a one-step growth cycle, but lower MOIs give us multi-step growth cycles.

Physical Measurements of Viral Particles:

- The following are methods to count the number of viral particles and are not measures of infectivity.

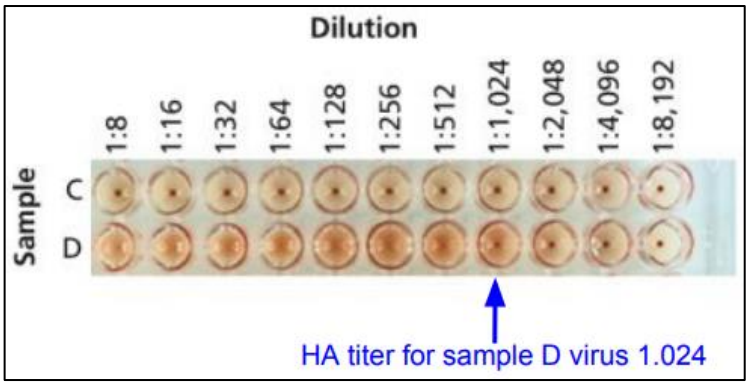
1. ***Hemagglutination:*** (based off of the adhesive capabilities of viruses/ e.g. attachment of viruses to a sugar such as sialic acid which is a receptor that viruses use to get into the cells.)

A type of assay used to determine if there is a virus present in a sample. Multiple wells are filled with equal volumes of normal blood, and serial dilutions are made of the sample containing the suspected virus (patient's sample -> can be a blood sample, stool sample, etc.) The sample dilutions are done in equal increments (1:8, 1:16, 1:32, etc.) and are mixed with the blood. If there is no virus present, the blood cells will clump together and sink to the bottom of the well forming a "*button*". (Blood cells w/o the virus.)



If there is a virus present, the blood cells will form a *lattice* and precipitate onto the walls of the well, so no button will form. *The point at which the sample containing a virus becomes too diluted to prevent the lattice from forming/ shows the first appearance of a button is called the* ***hemagglutination (HA) titer****.*

Shown here is a control sample-> Sample C, along with a sample that has a virus-> Sample D. The button in the infected sample (D) starts forming at a dilution of 1:1,024 so we say its HA titer is 1,024.



This is assay can be used to compare two people with the same viral infection. An example: A patient has HA titer of 64, and another patient has a HA titer of 1,024. Which patient has more viruses/ advanced infection? The patient with a HA titer of 1,024. Why? Because more dilutions are needed to prevent the lattice formation and initiate the formation of the button.

<u>The person with a higher HA titer has a more advanced stage of the infection/more viral particles in his body.</u>

2. ***Measuring Viral Enzymatic Activity:*** (e.g. measuring the viral enzyme activity of reverse transcriptase in retroviruses -HIV)

This technique attempts to approximate the number of viruses based on the activity of an enzyme unique to that virus. A solution cultivated with viruses is prepared, followed by adding the tagged substrate of the unique viral enzyme. The substrate is tagged with radioactive or fluorescent molecules. Detergents are also added to help fasten the process of substrate incorporation into the virus (by increasing viral permeability). Finally, measuring how well incorporated the tagged substrate is inside the cells (i.e. measuring enzyme activity) is done by measuring the amount of radioactivity incorporated. More enzymes-> more enzyme activity -> more viruses.

3. ***Serology:***

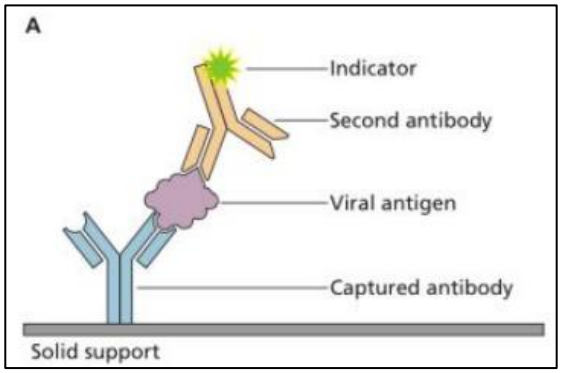
There are multiple serologic techniques that can be used to measure viral particles, the most famous one is **ELISA.**

Enzyme linked immunosorbent assay (ELISA):

-This technique uses enzymes, antigens, and tags in order to quantify viral particles in a sample. First serum (extracted through blood centrifugation to obtain plasma and then plasma is defibrinated) is taken from a patient to see whether they have a virus or not (serum is just plasma without the clotting factors). The serum is then incubated in a 96 plate/well with captured antibodies, attached to a solid support, that are designed to bind to a viral antigen. After the viral antigen binds to the first antibody, another antibody is added and binds to a different site on the viral antigen. This antibody is introduced with an indicator/signal (mostly fluorescent). Measuring the signals by using a plate reader gives us an idea about the presence of the viruses (no signal means no antigen means no virus).

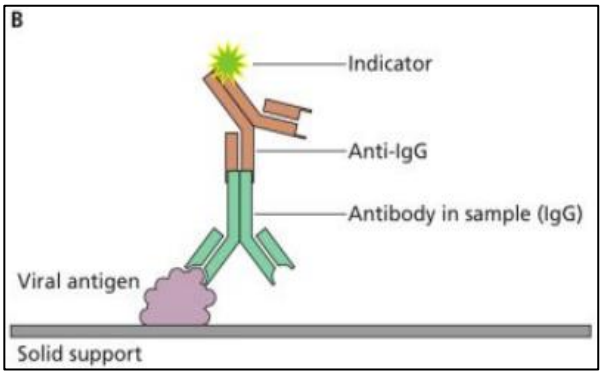
-A control of the viral antigen is initially provided (by the company that produces the ELISA) with another set of plates, and it contains several **known** dilutions of the virus. These dilutions are then plotted producing a standard curve also known as a calibration curve. This curve allows us to quantify the number of viruses in the original sample taken from the patient by simply comparing the dilution values to the ones plotted on the curve/control.

https://www.youtube.com/watch?v=6Ue1Hd3dyaQ Watch this video for a good visualization.



✳The ELISA test is used to test for the viral antigen. The same technique can also be used to see if the patient has antibodies for that viral antigen, so instead of having an antibody attached to the solid support, we simply attach the viral antigen instead.

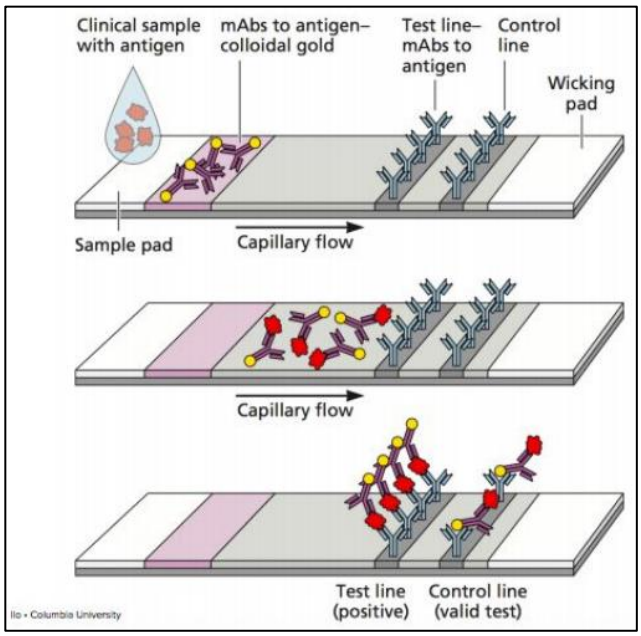
✳If the sample has antibodies in it, then binding occurs and one can detect this antibody by using a second antibody that has an indicator attached to it.



Lateral Flow Assay:

This test is another serologic test based off the principles used by ELISA, that is faster and can be done at home, without laboratory equipment or professional help.

★This test (LFA) uses a concept called **capillary flow** in order to move the antigens (red colored) towards the antibodies (purple colored) that are waiting to bind. In turn, these antibodies will bind to the antigens from the sample (assuming there is a viral particle) and then continue moving toward other the antibodies (blue colored) that are connected to the solid support. The test line of trapped antibodies is there to bind to the majority of the antibody-antigen complexes and shows a colored line indicating a positive test, and the second/control line validates the test to make sure it is not a false positive.



- The initial antibodies (purple colored) can sometimes move due to the power of the capillary flow and not necessarily due to the presence of antigens. They can get carried to the test line but getting carried to the control line is merely impossible. (test line crossed only-> false positive) And this is how the control line validates the test making sure it's not a false positive. So, in order for the test to be positive -> antibodies should pass both lines.

➔ If there are no antigens, the sample will move but the antibodies (purple colored) won't move with it simply because there are no antigens attached to the antibodies.

4. ***Nucleic Acid Tests***

The next set of tests examine a sample to see if there are pieces of viral nucleic acids.

Polymerase Chain Reaction:

Used when: A sample is containing a virus and with this amount of virus one can't tell the amount of DNA present. So, through amplification of this initial amount of DNA, one can have an idea about the actual amount of the virus.

This reaction is used to multiply a piece of **DNA** many times in a short period, using the same enzymes (*thermally stable* DNA polymerase, etc.) used for DNA replication. Instead of doing it in vivo this can be done in a test tube in a lab. Instead of using human DNA polymerase, a heat stable copy from a bacterium that lives in hot springs is used. This is done in order to use heat to unzip the DNA molecule, a substitution for helicase in normal cells. These are the steps:

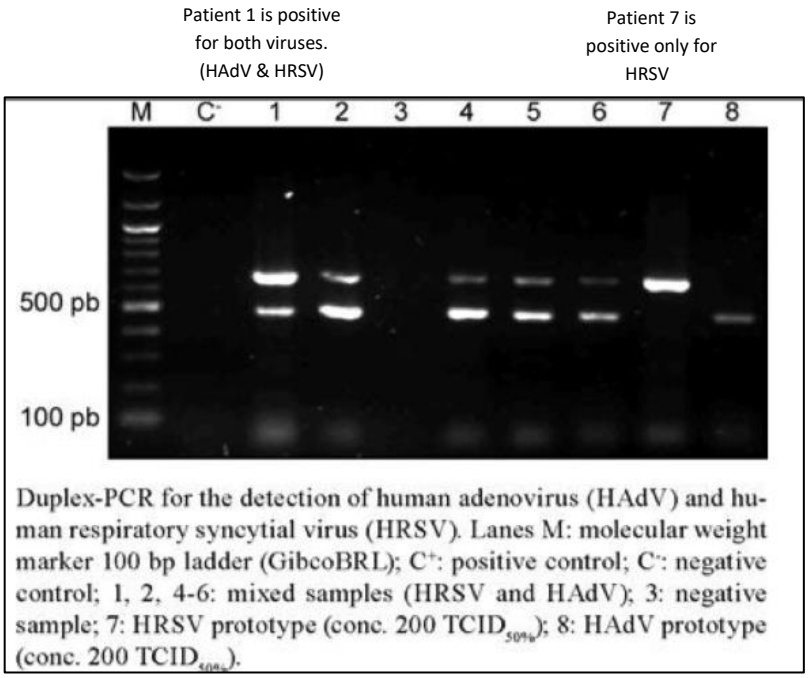
1. Choose a region of viral DNA (several 100 nucleotides)
2. This region of viral DNA is heated in a test tube with all the enzymes needed in DNA replication + nucleotides
3. A small sequence of primer is added (300-500 nucleotides). This sequence is unique to the viral DNA (this is so it doesn't bind to the host cells nucleic acids)
4. Now the enzymes begin working by themselves, replicating the sequence
5. These steps are repeated until a satisfactory amount of DNA has been produced

***Note**: each time this is done it is called a '**cycle**' and each cycle doubles the amount of DNA/RNA from the previous cycle (an exponential increase).

After doing PCR amplification, we run its product through gel electrophoresis in order to identify the virus that the patient has. *Electrophoresis is able to qualitatively show the presence of a virus, as well as roughly showing how many viruses the patient has, based on the band intensity.*

➔ As we can see, the more intense bands, the more copies of the nucleotide sequence. The left most column (Column C) contains water and is a negative control to show there is no contamination in the sample.

The PCR test is a qualitative/ semi-qualitative test.

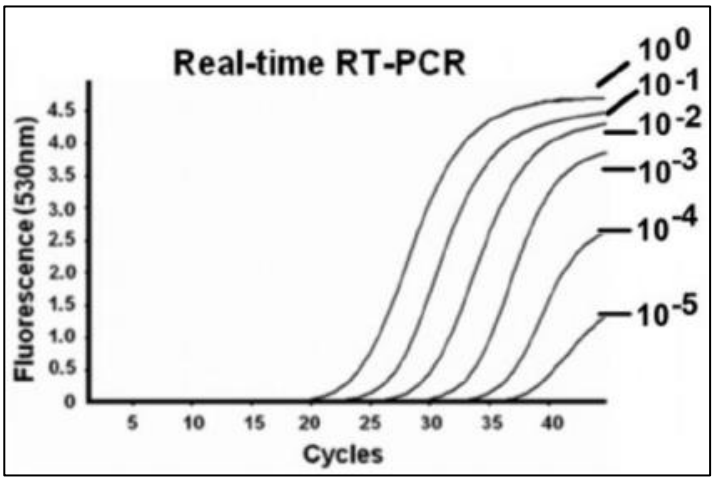


Duplex-PCR for the detection of human adenovirus (HAdV) and human respiratory syncytial virus (HRSV). Lanes M: molecular weight marker 100 bp ladder (GibcoBRL); C^+: positive control; C^-: negative control; 1, 2, 4-6: mixed samples (HRSV and HAdV); 3: negative sample; 7: HRSV prototype (conc. 200 $TCID_{50\%}$); 8: HAdV prototype (conc. 200 $TCID_{50\%}$).

Real-Time PCR:

◆Because gel electrophoresis is qualitative, a new type of PCR was developed to quantify results. The same technique is used, but the nucleotides that are put into the test tube are **fluorescent**. This means as the reaction goes through many cycles, more and more fluorescent nucleotides will be incorporated into the RNA and thus the sample will become brighter. A special camera then measures the fluorescence and is able to graph it over time. (As complementary DNA, from the **RNA**, is being synthesized, fluorescence simultaneously increases and that's why it's called real-time.) This approach is a strong **quantitative approach.**

➔ This graph can be used as is a comparison of different patients infected with the same virus (for example, Hepatitis C). According to the graph, the patient that is infected with more viral particles, their graph will appear faster on the fluorescence curve i.e. it will appear on earlier cycles. E.g. patient 10^0 is more infected with viral particles compared to patient 10^-4 since the patient's 10^0 graph appeared after 20 cycles rather than appearing after 33 cycles in patient 10^-4.



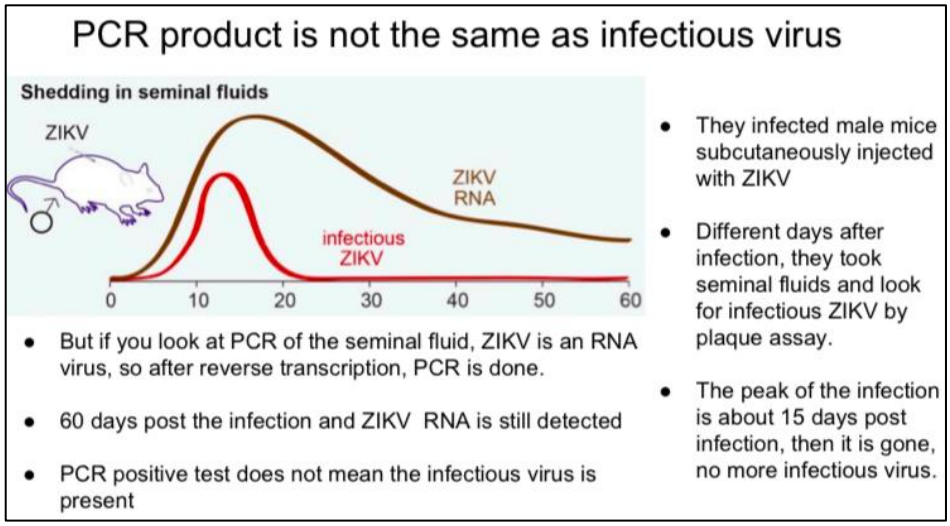
◊ IMPORTANT NOTE ◊: PCR and real-time PCR do not measure infectivity. They only show whether viral DNA/RNA is found in a sample or not. PFU is the gold standard for measuring infectivity.

➔ An important example:

Red curve: PFU

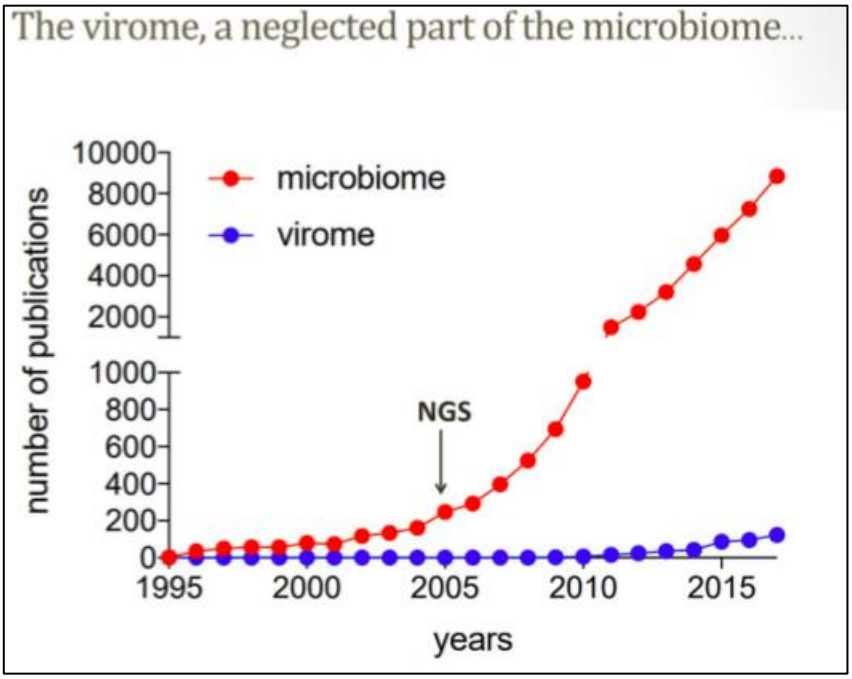
Brown Curve: PCR

E.g.: After 40 days, no PFU but still RNA is detected.



New Generation Sequencing (NGS):

◊ This method, also called *deep sequencing* or *high-throughput sequencing*, is a new technique that can sequence the **ENTIRE** genome of any organism in only 6 weeks, and only costs $1000 (to put into context the Human Genome Project took 13 years and many thousands of dollars to complete). Due to this revolutionary project, the number of publications on the human microbiome has grown exponentially since 2005. The human virome however has yet to show an increase in yearly publications.



☺ Last but not least, just a quick reminder that there are 10 bacteria and 100 viruses for every human cell in your body even in the none diseased state (not all viruses cause disease).

THE SECRET TO GETTING AHEAD IS GETTING STARTED ☺