

Virological Methods

Flint et al. Principles of Virology (ASM), Chapter 2

Overview

The most commonly used laboratory methods for the detection of viruses and virus components in biological samples can be broadly divided into 3 categories – those that measure virus **infectivity**, those that examine viral **serology** (that is, host antibody responses to the virus), and those that rely on **molecular methods**. One method which does not precisely fit into any of these categories is the use of imaging techniques to directly visualize virus particles.

Direct visualization of virus particles

Because viruses are submicroscopic, they cannot be assayed directly using the light microscope. Visualization of virus particles requires the use of the **electron microscope** (EM). The EM accelerates electrons to high energy, and focuses them using a magnet. The high energy of the electrons gives them a short wavelength, which is much smaller than that of viruses. Indeed, electron microscopes can visualize subviral molecules such as DNA or large proteins.

The high energy of the electrons used in the EM creates a problem in that they pass right through non-electron dense materials such as viruses. Thus, it is necessary to coat or stain samples for EM analysis. This process is called “shadowing” and it involves the deposition of a thin layer of a heavy metal such as osmium on the surface of the sample to be analyzed. One particularly useful aspect of EM analysis is that it allows one to directly count the number of virus particles within a sample – regardless of whether or not those particles are actually infectious.

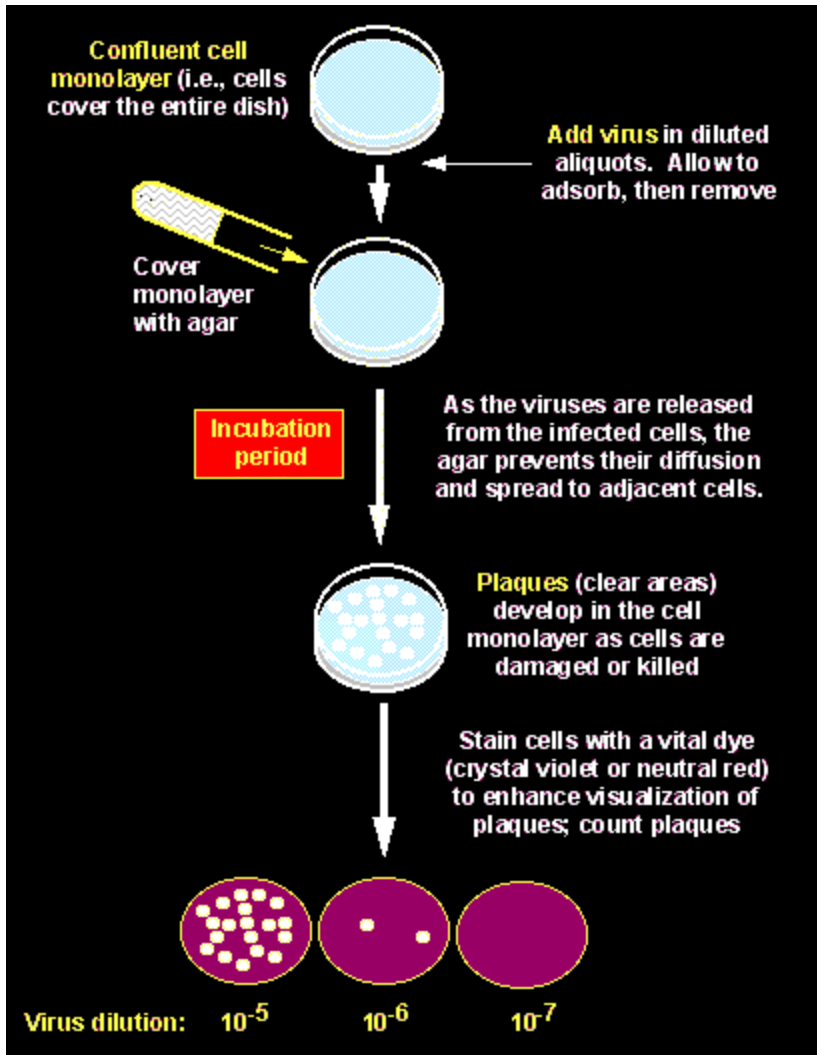
One problem of the shadowing process is that it tends to result in sample desiccation, and in some loss of structural information – particularly for enveloped viruses. As a result, the procedure of **cryoEM** analysis was developed. In this case, the virus sample is rapidly frozen onto an EM grid in such a way that it becomes embedded in a thin sheet of vitreous ice (ice that does not contain any large crystals). This process retains some moisture in the virus, and it results in enhanced preservation of viral structures. The virus particles are then visualized in the microscope at very low temperature, to prevent thawing. Visualization works because of the contrast in the electron density of the virus sample, versus that of the surrounding water matrix.

Other methods for visualization of viral proteins include X-ray crystallography (effective with large proteins) or NMR (effective with small proteins or peptides). Computer methods can be used to reconstruct images of the actual virus particle, based on an understanding of the underlying structures involved.

Measurement of Virus Infectious Units

To study and quantitate virus infectivity in the laboratory, animal viruses must be propagated in cells and cell lines. One very widely used assay is the **plaque assay**. This method is very similar to the procedure used to titer bacteriophages, and it relies upon (1) the use of confluent monolayers of cells which are susceptible to the virus, (2) the induction of a visible cytopathic effect by the virus (i.e., cell killing or cell damage) and (3) the use of a semisolid overlay (usually agar) which prevents virus diffusion from one infected cell to other nearby cells. As a result, small round plaques (clear areas) form in the cell monolayer as the virus replicates. These plaques can be visualized by staining the cells with a vital dye like crystal violet or neutral red.

Plaque Assay



Variations on the plaque assay include the fluorescent-focus assay and the infectious center assay. These assays are helpful in titering viruses that do not kill their host cells.

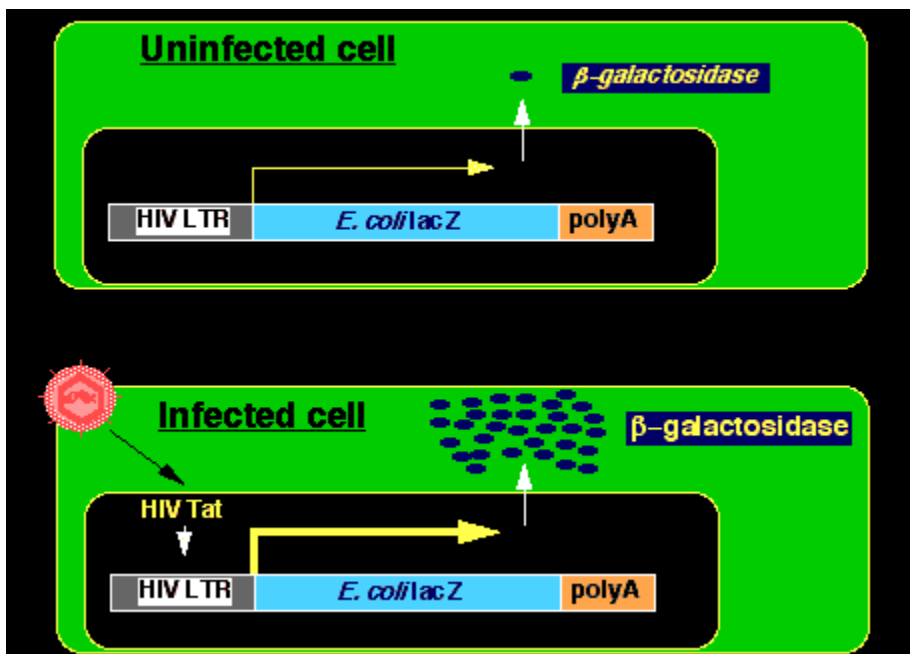
The **infectious center assay** allows one to determine the fraction of cells within a culture that are infected with virus. In this case, the infected cells are suspended, counted, and plated onto monolayers of susceptible cells, which are then overlaid with agar. The number of plaques which form in the indicator cells provides a measure of the number of virus-infected cells in the original culture.

In contrast, the **fluorescent-focus assay** does not rely on the induction of cell death (either in the infected cells or the indicator cells). Rather, it relies upon the use of antibody staining methods to detect virus antigens within infected cells in the monolayer. These infected cells are then visualized using a fluorescent label on the virus-specific antibody, and counted.

A related method is the use of indicator cells, which contain a **virus-inducible reporter**

gene. Examples of such systems include the MAGI cell system for detection of HIV infection. In this case, HIV-susceptible monolayer cells were genetically engineered to contain a reporter gene cassette comprising the viral long terminal repeat (LTR), placed upstream of the gene encoding *E. coli* β -galactosidase (*lacZ*). Expression of the *lacZ* gene is therefore dependent upon the transcriptional activity of the HIV LTR. Under normal circumstances this activity is weak and little β -galactosidase is produced (too little to detect). However, if the cell becomes infected by HIV-1, the virus will produce its transcriptional activator protein, Tat, and this will upregulate the activity of the viral LTR. As a result, expression of *lacZ* is greatly enhanced and one can now detect β -galactosidase using a chromogenic substrate such as X-gal.

Principle of the MAGI assay for HIV



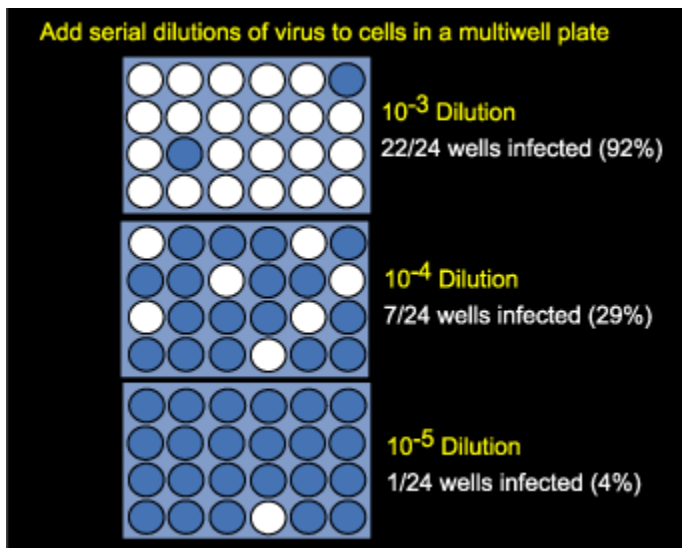
Assays analogous to the MAGI assay include the use of a green fluorescent protein (GFP) as an indicator gene, and the development of reporter cell lines for other viruses. Strategies for reporter gene cassette include not only virus-inducible promoters like the HIV LTR but also recombinant proteins, which can be cleaved by specific virus proteases in order to generate detectable enzymes or chromogens.

Other assays for virus infectivity include the **transformation assay**, which can be used to determine the titers or the biological activity of non-plaque forming viruses that have the ability to cause cellular growth transformation. Examples of such viruses include retroviruses (.e.g., the Rous sarcoma virus).

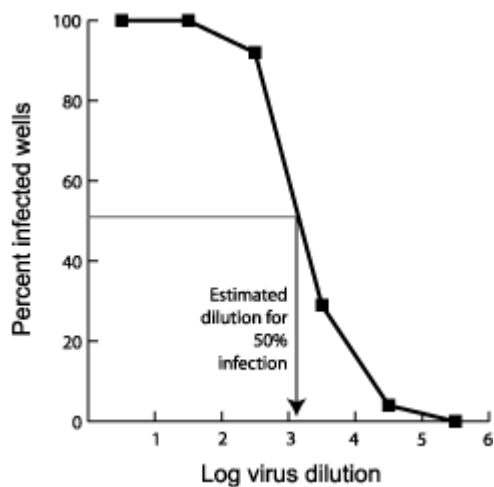
Finally, the **endpoint dilution assay** can be used to determine titers of many viruses, including viruses, which do not infect monolayer cells (making it impossible to use

plaque assays). In this case, one adds serially diluted aliquots of virus to cultured cells, which are typically plated into multiwell plates. One then allows the cells to grow for some fixed period of time, after which the wells are scored for the presence of viral replication. This is most often done by analysis of virally induced cytopathic effect, but it can also be done by analyzing cells or cell culture fluids for the presence of viral proteins or enzymes (e.g., for HIV-1, one can either measure reverse transcriptase activity using an enzymatic assay or one can measure viral p24 core protein production, using an ELISA assay).

Endpoint dilution assay



Endpoint dilution assays provide a widely used basis for the calculation of virus titers. The basic way in which this is done is shown below:



Typically, the 50% infectious dose ($TCID_{50}$ or tissue culture 50% infectious dose) is

calculated using a mathematical analysis of the data. Popular methods include that of Reed and Munch, or that of Spearman and Karber. The former is outlined below.

Reed & Munch endpoint calculation method

Note that the textbook (Flint et al.) has an error in the Reed-Munch formula; the formula below is the correct one!

Formulae

$$I = \left\{ \begin{array}{l} \frac{(\% \text{ of wells infected at dilution above } 50\% - 50\%)}{(\% \text{ of wells infected at dilution above } 50\% - \% \text{ of wells infected at dilution below})} \end{array} \right\}$$

$$50\% \text{ endpoint titer} = 10^{\log \text{ total dilution above } 50\% - (I \times \log h)}$$

Definitions

I = Interpolated value of the 50% endpoint (*also known as the proportional distance*)

h = dilution factor

Example

Dilution	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Positives	24	22	7	1	0
Wells inoculated	24	24	24	24	24
% infected	100	92	29	4	0
	= [(92-50)/(92-29)]				
	= [42/63]				
	= 0.66				
50% endpoint titer	= $10^{-3 - (0.66 \times 1)}$				
	= $10^{-3.66}$				

The calculation of virus titers, and their comparison with the total numbers of virus particles (as determined by EM analysis) allows one to determine the particle-to-infectivity ratios for viruses. In many cases, it emerges that the great majority of virus particles are in fact defective – that is, they are incapable of replication.

Particle/plaque forming units for selected animal viruses

Virus	Particle/PFU ratio
Poliovirus	30 - 1000
Adenovirus	20 - 100
Herpes simplex virus	50 - 200
Papillomavirus	10,000
Semliki forest virus	1 - 2

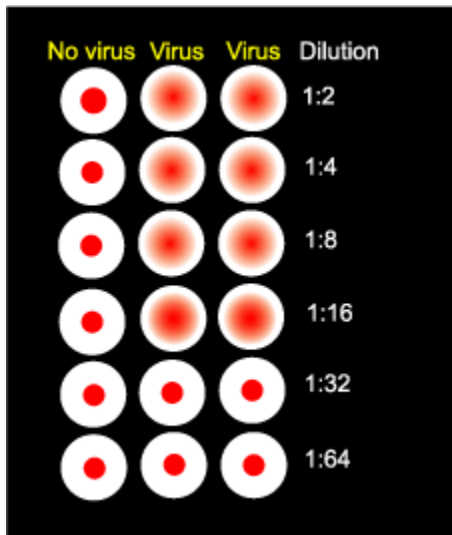
Quantitation of Virus Particles and Components

Thereof

As noted previously, EM analysis can be used to directly visualize virus particles. Another method that has been widely used to measure virus particles is the **hemagglutination assay**. This assay takes advantage of the fact that many viruses contain proteins that can bind red blood cells (erythrocytes). Examples of viral proteins with the ability to bind to red blood cells include the influenza virus hemagglutinin (HA) protein, which binds to sialic acid residues on the cell surface.

Normally, red blood cells will fall to the bottom of a culture well, forming a sharp dot. However, if viruses are present, the red cells become bound to the virus particles in a lattice or network (this happens because a single virus can bind multiple red blood cells). This lattice then coats the well. The assay is fast (30 minutes).

Example of hemagglutination assay



Viral enzyme assays

As noted previously, one can measure the activity of virally-encoded enzymes such as reverse transcriptase (retroviruses, hepatitis B virus) or viral proteases (encoded by many viruses, and used in protein maturation; these are useful because they have unique cleavage specificities that differ from those used by cellular proteases). Other unique viral enzymes may also be possible substrates for assay development

Serologic Methods

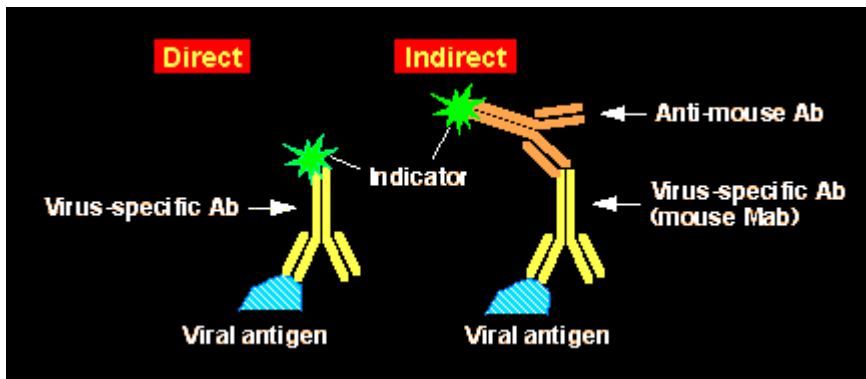
A variety of serologic, or antibody based, methods can be used to detect viral proteins or host immune responses to viral infection.

Virus neutralization is a method in which antibodies are added to a virus preparation, and the infectivity of this preparation is then measured using indicator cells. Typically, a subset of the antibodies produced against any given virus will have the ability to interfere with the interaction between the virus and its host cell receptor. Such antibodies will have the ability to neutralize the infectivity of the virus. Other antibodies may react with the virus, but may fail to prevent it from infecting its target cell. Such antibodies would not have neutralizing activity.

Neutralization assays have been very useful in defining **viral serotypes**. For example, poliovirus serotypes were defined serologically, using neutralization assays (i.e., poliovirus type 1 is neutralized by antiserum to type 1 viruses, but not by antiserum to types 2 or 3, and so on). The definition of viral serotypes is a useful tool in terms of classification, and it is also of potential significance in the development of vaccines since one may need to include examples of all the virus serotypes in order to develop an

effective vaccine. Thus, both the live-attenuated (“Sabin”) and the whole-killed (“Salk”) poliovirus vaccines contain mixtures of all three virus serotypes.

Immunostaining. This is a method in which antibodies are used to detect viral proteins in infected tissues or cells. Two basic methods are used. In direct immunostaining, the virus-specific antibody is directly labeled with an indicator molecule, so as to permit its visualization (the indicator molecule is usually either an enzyme such as horseradish peroxidase or a fluorescent molecule such as fluorescein). In indirect immunostaining, the virus-specific antibody is detected using a labeled secondary antibody directed against the first antibody (e.g., a labeled anti-mouse antibody if the initial antibody was a murine monoclonal antibody).

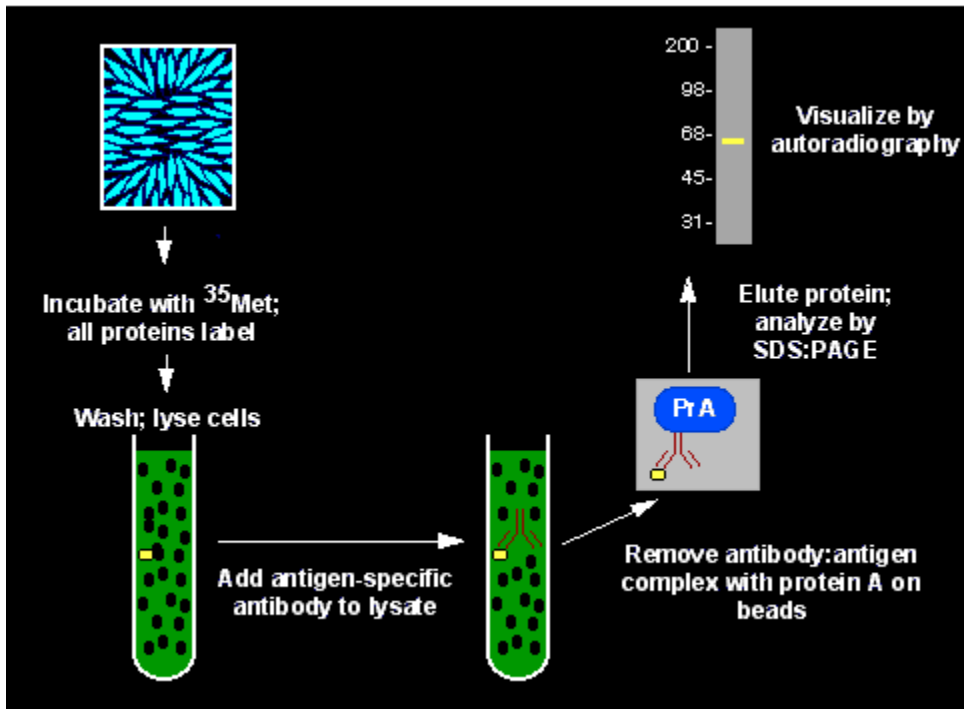


Direct and indirect immunofluorescent staining methods

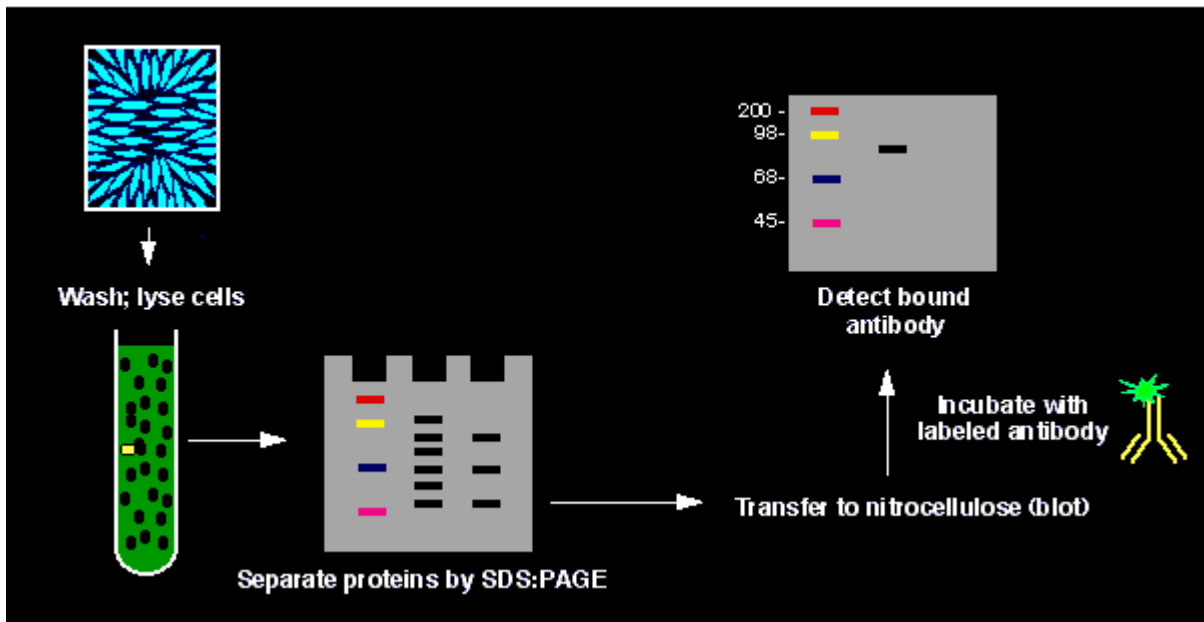
Immunoblotting and immunoprecipitation. These methods allow one to detect specific viral proteins in lysates from infected tissues or cells. In the case of immunoprecipitation, one typically pre-labels cells (and proteins) with radioactivity. The cells are then lysed, and the labeled proteins are precipitated out of solution using a specific antibody. The antibody:protein complex is then captured and purified, after which the bound protein is released, for analysis by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

In immunoblot analysis, the proteins are not pre-labeled with radioactivity. In stead, the whole cell extract is subjected to SDS-PAGE analysis, so as to separate out the proteins based on their size and other physical properties. The proteins are then transferred to a solid membrane support (often nitrocellulose), either by capillary action, pressure or by electrophoresis. Membranes can then be probed with specific antibodies, and the bound antibodies can be visualized using highly sensitive indicator molecules (for example, enzymes such as horseradish peroxidase).

Immunoprecipitation method



Immunoblot method

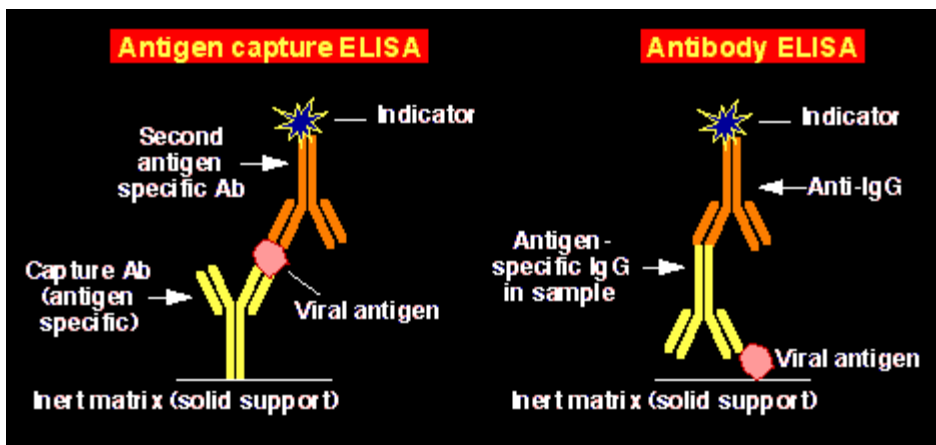


Enzyme-linked immunosorbent assay (ELISA).

ELISA assays allow the detection of viral antigen or antibody, using a solid-phase assay system. For **antigen capture ELISA** (in which virus protein is measured), a solid matrix (plastic dish) is coated with a capture antibody specific for the viral protein of interest.

The virus-containing test sample (e.g., supernatant from a virally-infected cell culture) is then added to the well. After allowing time for the protein to bind to the antibody, the well is washed and a second antibody is then added. This detection antibody is also specific for the virus protein being assayed, and it has an indicator molecule attached to it to allow for quantitative analysis of binding.

Antibody ELISA is a very similar method, except that in this case, one is measuring the amount of antibody directed against a specific virus protein in a sample of serum from an infected person or animal. In this case, the plastic dish is precoated with the virus protein. The antibody-containing test sample (serum) is then added. After incubation and washing, the bound antibody is detected using a secondary antibody that is usually directed against the constant region (Fc) of the specific antibody isotype of interest (most commonly, IgG).



Nucleic acid detection.

Methods for the detection of viral nucleic acids in clinical and laboratory specimens include Southern blot analysis, in situ hybridization, polymerase chain reaction (PCR) and the use of gene arrays. **Southern blot analysis** was developed in the 1970s, and it involves the fractionation of DNA with restriction enzymes, followed by transfer of DNA to a solid membrane support and detection of virus-specific sequences using radiolabeled viral nucleic acids. **Northern blot analysis** is an analogous method for the detection of RNA sequences.

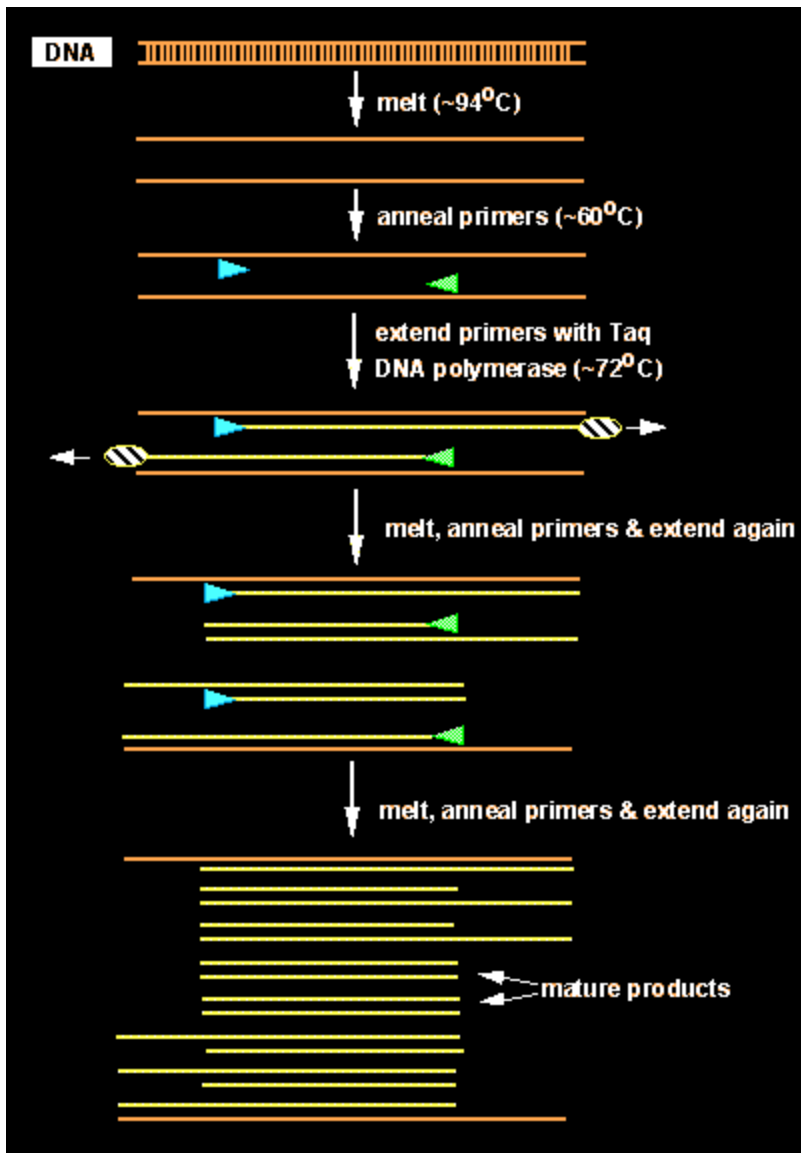
Nucleic acid probes can also be used to detect viral sequences within tissues or individual cells, by using the method of **in situ hybridization**.

One of the most widely used methods for detection of viral nucleic acids is the **polymerase chain reaction (PCR)**. In this method, specific oligonucleotides are used to amplify fragments of viral nucleic acid from cell or tissue samples. The method uses a thermostable DNA polymerase, and multiple cycles of amplification, which comprise 3 steps – (1) melting of the double-stranded DNA template, (2) annealing of the

oligonucleotide primers to the template and (3) extension of the primers by the thermostable DNA polymerase, thus generating new DNA chains.

PCR analysis can also be adapted to the analysis of viral RNA, by including an initial step in which the RNA is converted into DNA, using a reverse transcriptase enzyme. This is referred to as reverse transcription (RT)-PCR.

Outline of the PCR process (first few cycles of amplification)



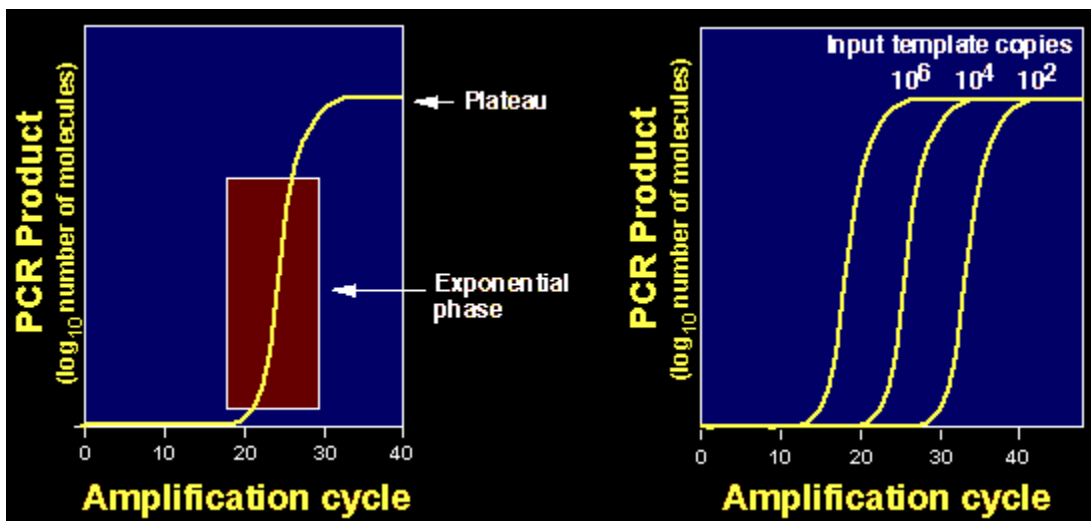
Quantitation of the PCR process. In theory, the PCR process should result in unlimited exponential amplification of the DNA segment of interest. However, this idealized situation does not occur in reality.

The typical reaction profile of a PCR reaction is shown below. It can be observed that the relationship between the log of the amount of PCR product produced and the number of cycles of amplification performed is linear only for a finite period. In later cycles, a plateau is reached as the reaction components become depleted (panel A).

The second important point that can be appreciated is that, during the exponential phase of the amplification reaction, the amount of PCR product produced at any given cycle is related to the amount of input (template) DNA (panel B). Thus, PCR can be used to directly quantitate the amount of PCR product in a sample.

A: PCR amplification profile

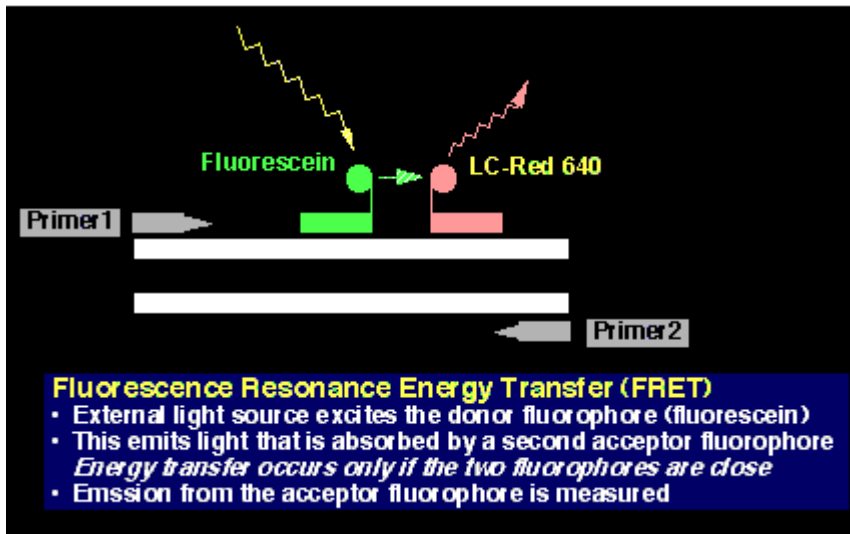
B: Amplification VS amount of input DNA



The extreme sensitivity of PCR has made it very useful in terms of viral diagnosis, and the ability to use PCR as a means to quantify the amount of viral nucleic acid with clinical or laboratory specimens has proven very valuable in allowing investigators to measure viral replication.

Methods for the quantitative analysis of nucleic acids using PCR include so-called “real time” PCR methods. This involves the direct measurement of the amount of PCR product produced while the reaction is in progress. Mathematical analysis of the data, and comparison to control reactions containing known amounts of template, allow one to then calculate the amount of input DNA in the initial reaction.

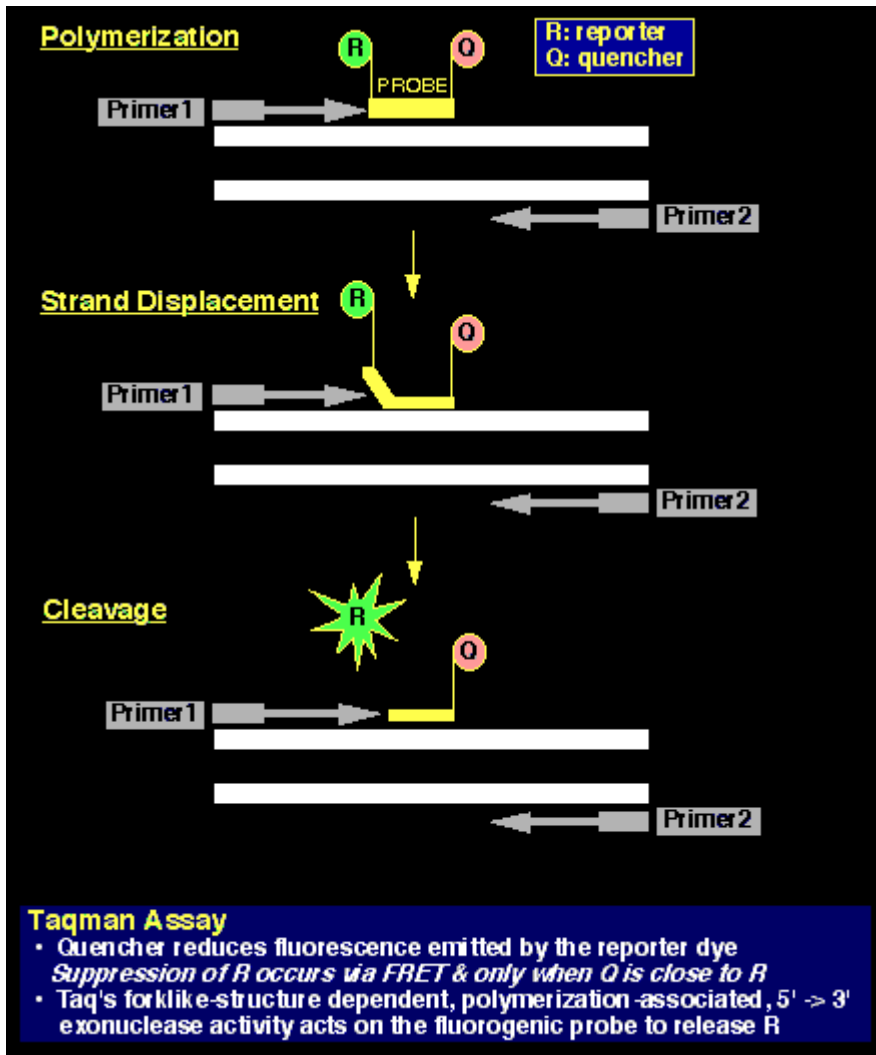
Real-time PCR analysis is performed by taking advantage of a process known as fluorescence resonance energy transfer (FRET). One way in which FRET has been used for quantitation of PCR reaction product is shown below. In this example, two oligonucleotide probes are generated, each of which is complementary to the same strand of a particular PCR product.



The two probes each bear a chosen fluorophore. One of the probes contains a fluorophore that is excited by an external light source, causing it to emit light of a different wavelength. That emitted light in turn excites the second fluorophore, causing it to emit light. The light emitted by the second fluorophore is then measured.

The trick here is that the process of fluorescence energy transfer only occurs if the two fluorophores are in very close proximity. That can only happen if the two oligonucleotides have both become annealed to the same DNA molecule (PCR template), but it will not happen otherwise.

Real-time PCR



Finally, one powerful technology which is becoming increasingly useful in the analysis of viral infections is that of **DNA microarray** technology (aka gene chips). In this method, a high density DNA array is generated, comprising millions of different oligonucleotides spotted onto a solid support. Labeled sample DNA (usually biotinylated) is then added to this microarray, and allowed to anneal. After extensive washing, the bound sample DNA is detected using a fluorescent probe (e.g., streptavidin-phycoerythrin).

DNA microarray can be used to probe for specific point mutations in the human genome (e.g., disease associated mutations, or mutations associated with enhanced susceptibility to certain viral infections). It can also be used to detect viruses, and even to detect specific mutations within virus populations – for example, mutations associated with resistance to antiviral drugs (see below).

DNA microarray technology

