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Chapter 7

Detection and Diagnosis of Viral Infections

Viruses have evolved alongside humans for as long as both have existed. "Filterable viruses" were classified as a separate pathogen upon their identification in 1898, but infectious diseases have been characterized throughout history by the clinical conditions they have caused, despite that the doctors of the time had no idea of the existence of microscopic pathogens. Hippocrates (460–377 BC), the father of modern medicine, described several illnesses characteristic of viral diseases, including influenza and poliomyelitis. The ability to definitively identify a specific virus as the cause of an illness has only become possible within the last 100 years.

As described in Chapter 1, "The World of Viruses," bacteria can be viewed under a light microscope, but viruses are too small to be visualized with light microscopes. Consequently, the first efforts to identify specific viruses relied upon **serology**, the analysis of the protein antibodies found in blood that the immune system synthesizes against pathogens. **Tissue culture**, the ability to grow tissues and cells outside of a living organism in a controlled environment, was invented and refined in the first half of the 20th century. This led to the propagation of viruses using cell culture and the detection of the pathogenic effects that viruses exert upon cells. Both serology and tissue culture have been refined and are still vital techniques for the diagnosis of viral infections. Advances in molecular biology have also accelerated our ability to conclusively identify a virus.

The detection of a virus as the cause of an illness is important for many reasons. Several viral infections result in similar clinical symptoms, and viruses with serious effects need to be identified early in order to prescribe the best treatment. Likewise, the infection of high-risk groups, such as transplant recipients, pregnant women, or immunocompromised individuals, needs to be monitored so that critical sequelae can be addressed. The development and availability of antiviral drugs is increasing, and proper diagnosis ensures an effective treatment is prescribed. The typing of viruses is also effective in determining subtypes or strains of viruses, including those that are resistant to certain drugs or are more likely to cause cancer. In the field of epidemiology, most case definitions rely upon laboratory confirmation of the specific virus to confirm a case. Proper diagnosis ensures that accurate surveillance takes place and adequate control measures are instituted during epidemics. It also ensures the safety of transplanted human tissues and safeguards the blood supply. This chapter discusses commonly used techniques for the detection and diagnosis of viruses in clinical samples. Many of these methods are staples in virology research laboratories, as well.

7.1 COLLECTION AND TRANSPORT OF CLINICAL SPECIMENS

For identifying a specific virus, the type of specimen obtained depends upon the type of virus. The specimen will be isolated from the location of infection for viruses that establish localized infections (Table 7.1). For instance, influenza virus is readily detected from nasopharyngeal swabs, and herpes simplex viruses can be isolated from the oral or genital lesions that these viruses cause (Table 7.2). Viruses that establish systemic infection may be isolated from several different sources, depending upon the virus. The site of pathology is often a good place to start, although the virus may be present in the blood as well. For example, hepatitis B virus and hepatitis C virus infect hepatocytes (liver cells) but are detectable in serum.

The choice of diagnostic test will also depend upon the stage of infection. A person's viral load is highest during acute infection but may drop to undetectable levels as the infection is cleared. On the other hand, it takes weeks for antibodies to develop during the primary response against a virus (Fig. 7.1). As described in Chapter 6, "The Immune Response to Viruses," IgM is the antibody isotype that is first produced by plasma cells against a pathogen, but the higher-affinity IgG isotype begins being secreted by plasma cells later during infection and during secondary responses. Therefore, the choice of diagnostic test will depend upon the patient's stage of infection. Tests for the virus itself are best performed before or while symptoms are present. The levels of IgM versus IgG antibodies against a virus can be used to help determine if the infection recently occurred, but neither of these will be present at the beginning of a primary infection.

Care must be taken in the collection, storage, and transport of clinical specimens. Blood is collected into appropriate tubes, depending upon whether cells, serum, or plasma is required for the diagnostic test. Tubes containing sodium heparin or EDTA as an anticoagulant block the clotting of blood and are used to obtain white blood cells (**leukocytes**) or **plasma**, the liquid fraction that remains when blood is

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TABLE 7.1 Types of Specimens Collected for Viral Diagnosis

Site (or type) of illness	Possible viral cause	Types of specimens collected
Respiratory tract	Adenovirus	Nasopharyngeal swab, nasal aspirate, nasal swab, nasal wash, throat swab
	Cytomegalovirus	
	Enterovirus	
	Herpes simplex virus	
	Influenza virus	
	Parainfluenza virus	
	Respiratory syncytial virus	
Gastrointestinal tract	Adenovirus	Stool, vomit
	Rotavirus	
	Norwalk virus	
Skin (rash)	Coxsackie A virus	Biopsy, Tzanck smear
	Herpes simplex virus	
	Varicella zoster virus	
Eye	Adenovirus	Conjunctival swab, corneal swab
	Cytomegalovirus	
	Enterovirus	
	Herpes simplex virus	
	Varicella zoster virus	
Central nervous system (Meningitis, encephalitis)	Arboviruses (many)	Cerebral spinal fluid, stool, biopsy (or autopsy), throat swab, blood
	Coxsackie A virus	
	Coxsackie B virus	
	Dengue virus	
	Enterovirus	
	Herpesviruses	
	Lymphocytic choriomeningitis virus	
	Measles virus	
	Mumps virus	
	Poliovirus	
	West Nile virus	
Genital infections	Herpes simplex virus	Cervical swab, urethral swab, vesicle fluid, Pap smear, Tzanck smear
	Human papillomavirus	

centrifuged to pellet blood cells. **Serum** is obtained by allowing blood to clot and then centrifuging the clot, leaving behind the liquid portion of the blood (Fig. 7.2). The difference between plasma and serum, therefore, is that plasma contains clotting factors that are part of the clot when serum is obtained. Antibodies and virus/virus antigen will be found in serum and plasma, although virus could also be found in the leukocytes, if they are a target of the virus. For collecting fluid from skin lesions, a sterile swab is used to collect the fluid and cells from a lesion that has been opened, and then the swab is placed in a special transport medium. The same transport medium is used for nasopharyngeal swabs, cervical swabs, rectal swabs, or throat swabs. Stool is collected into a clean, leak-proof container.

TABLE 7.2 Specimens Collected for Select Human Viruses

Virus	Type of specimen collected for identification	Tests for virus?	Tests for antibodies?
Influenza	Nasopharyngeal swab, nasal aspirate, nasal swab, nasal wash, throat swab	Yes	No
Norwalk virus	Stool, vomit	Yes	No
Hepatitis viruses	Serum	Yes (HBV, HCV)	Yes
Herpes simplex virus	Scraping from site of infection: oral mucosa, genital mucosa, conjunctiva or cornea	Yes	No
	Serum	No	Yes
Human immunodeficiency virus	Serum	Yes	Yes
	Saliva	No	Yes
Human papillomavirus	Pap smear or cervical swab	Yes	No
Rabies virus	Cerebral spinal fluid, serum	No	Yes
	Saliva	Yes	No
Ebola virus	Serum	Yes	Yes
West Nile virus	Serum, cerebral spinal fluid	Yes	Yes

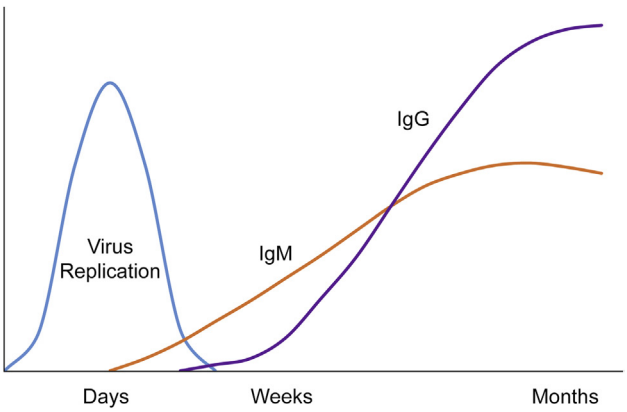

FIGURE 7.1 Considerations for choice of diagnostics. Diagnostic tests are available that test directly for the presence of virus, which occurs during active infection, or antiviral antibodies, which take weeks to develop and continue for months following infection. IgM is produced during a primary response, while IgG takes additional time to develop and will be produced during secondary responses. The choice of test depends upon the state of the infection and whether virus or antibody is likely to be present at that time.

The susceptibility of viruses to environmental factors can be an issue for diagnostic tests that rely upon "live" virus. As mentioned in Chapter 5, "Virus Transmission and Epidemiology," most viruses will become noninfectious after being exposed to extended periods of heat. Although nucleic acids may be able to be recovered from these samples, infectious virus will not be present. To prevent virus inactivation, samples other than blood that must be transported to diagnostic laboratories are refrigerated during shipment, or frozen at −80°C and shipped on dry ice if the transport will take 3 days or more. However, some viruses are not stable when frozen, including varicella zoster virus, respiratory syncytial virus, measles, and human cytomegalovirus. These viruses must be frozen in a special transport medium to prevent their inactivation. Much emphasis is placed upon the efficacy of the diagnostic test itself, but no assay can provide meaningful results if the specimen has not been collected, stored, and transported with care.

7.2 VIRUS CULTURE AND CELL/TISSUE SPECIMENS

Methods for detecting viruses are either direct or indirect methods. **Direct methods** assay for the presence of the virus itself, while **indirect methods** observe the effects of the virus, such as cell death or the production of antibodies by the infected individual. Tissue culture is a way to identify a virus based upon the effects of the virus upon the cells. It is also a way to amplify virus if a larger sample is needed for other diagnostic tests, since viruses require cells to replicate.

Tissue culture, also known as **cell culture** when cells are grown specifically, involves maintaining living cells or tissues in a controlled environment outside a living organism. The cells are housed in plastic flasks or bottles and bathed in a liquid growth medium that contains nutrients and supplements (Fig. 7.3A). These cultures are grown in

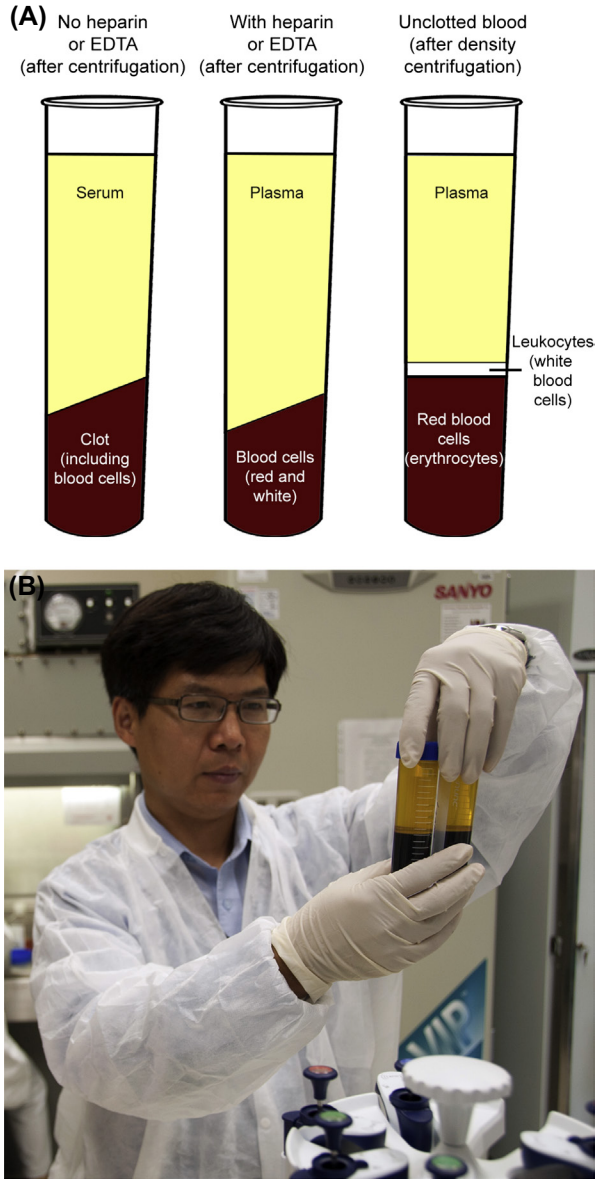

FIGURE 7.2 The constituents of blood. Different diagnostic tests require different components of blood. (A) In a tube without anticoagulants, a clot will form that can be centrifuged to the bottom of the tube. The acellular liquid fraction is serum. A tube with heparin or ETDA as coagulants prevents clotting factors from working. The cellular fraction is centrifuged, and the liquid fraction—with clotting factors—is known as plasma. If unclotted blood is centrifuged slowly in a special medium to separate the blood by density, a thin white line of leukocytes, called the buffy coat, separates the plasma and red blood cell layers. In this case, about 55% of the total blood is plasma, 45% are erythrocytes, and <1% are leukocytes. Antibodies are found in serum or plasma. (B) A researcher examines two blood density centrifugations. Note the thin layer of leukocytes in between the plasma and red blood cell layers. *Photo courtesy of FDA / Michael J. Ermarth.*

an incubator set to body temperature (37°C). A **cell line** is a set of cells that have been isolated from a tissue or organ fragment (Fig. 7.3B and C). Cell lines can be **finite** or **continuous.** Finite cell lines will only undergo mitosis a limited number of times, while continuous cell lines are immortal and will proliferate indefinitely. This characteristic is usually a result of genetic mutations. Cell lines derived from tumors, which have lost control of regulating the cell cycle and proliferate indefinitely, can also result in continuous cell lines. The choice of a finite versus continuous cell line for propagating viruses in culture will depend upon the virus that is needed to be isolated. Additionally, the cell line must be one that expresses the cell surface receptor specific for the virus and be permissive to infection; otherwise, the virus will not be able to attach and replicate within the cell line.

Cell cultures must be grown using aseptic technique and sterile conditions, otherwise bacteria and fungi that get into the cell culture will grow profusely in the rich growth medium and contaminate the culture. A sterile environment is provided by a **biological safety cabinet (BSC)**, which is different from a fume hood that is used when working with chemicals. A BSC uses a fan to filter air through **high-efficiency particulate air (HEPA)** filters, which filter out bacteria, fungi, spores, and viruses to generate sterile air. Particles of 0.3 μM in size are the most penetrating through the filters but are still removed with 99.97% efficiency. There are three different classes of BSCs, designated class I, class II, and class III, that provide varying levels of protection to the worker and to the biological material being manipulated. Class I BSCs act much like chemical fume hoods, except that the air is filtered through a HEPA filter before it is released to the environment. The worker and environment are protected, but the biological material is exposed to nonsterile air from the environment so these are not used for cell culture (Fig. 7.4A). Class II BSCs are most often used, as they afford protection to the worker, the biological material, and the environment. The air entering the BSC from the front is sucked into a grille to prevent it from contaminating the working surface, which is constantly bathed in HEPA-filtered air to ensure a sterile working environment (Fig. 7.4B and D). Class II BSCs rely on the **laminar flow** of air, which means that the BSC creates an uninterrupted flow of air in a consistent, uninterrupted stream. As long as the work is performed within the stream of air, then the material will remain sterile. The air is also sent through a HEPA filter before being released to the environment. Class II BSCs are sufficient to work with cells and the majority of viruses. Some dangerous pathogens must be manipulated within a class III BSC, which is air-tight to prevent any exposure of the virus to the worker, who must use the heavy-duty rubber gloves that are built into the BSC to work with the pathogen (Fig. 7.4C). The air leaving a class III BSC is passed through two HEPA filters, or a single HEPA filter and incinerator, to ensure the pathogen does not enter the environment.

Appropriate levels of safety must be taken when working with viruses. Rhinoviruses cause colds, and so the effects of accidentally being exposed to rhinovirus are minimal and self-limiting. Other viruses, such as viruses that cause hemorrhagic fevers, can lead to deadly effects if someone is

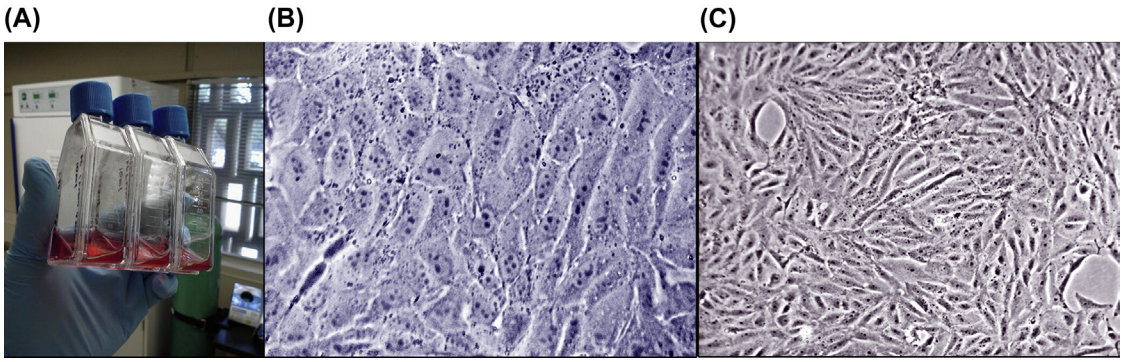

FIGURE 7.3 Cell culture. (A) Three cell culture flasks containing living cells and culture medium (pink). (B) MDCK cells, the preferred cell line for isolating influenza A and B viruses. (C) Vero cells, which are susceptible to infection with herpes simplex viruses, poliovirus, Coxsackie B virus, respiratory syncytial virus, mumps virus, rubella virus, SARS-CoV, and lymphocytic choriomeningitis virus, among others.

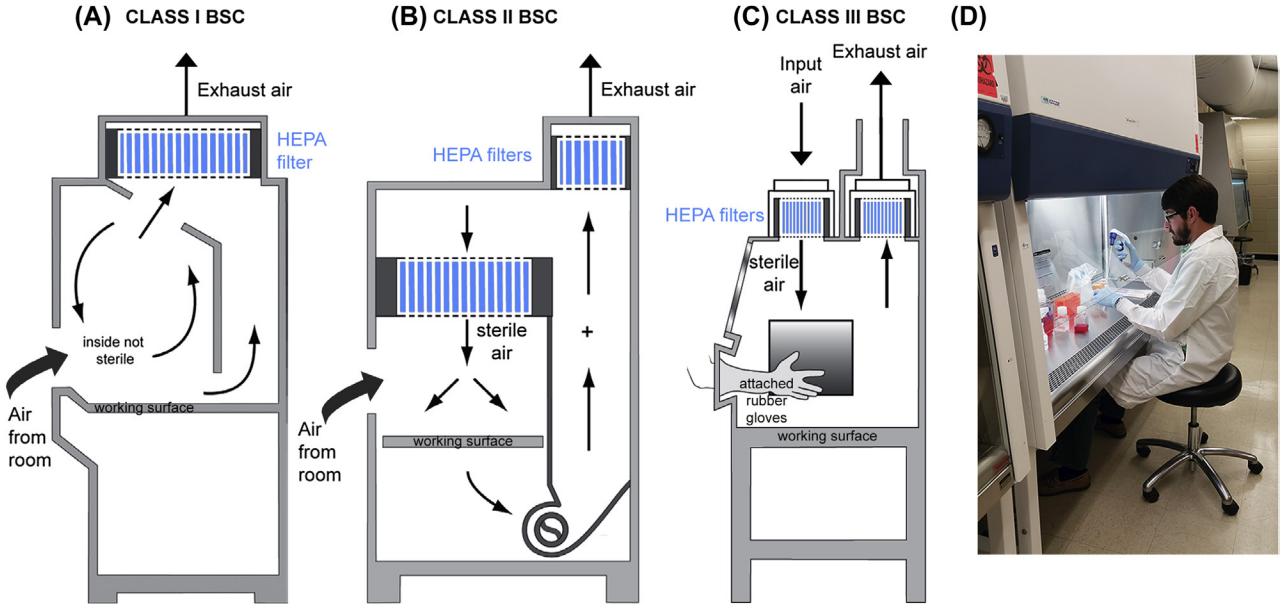

FIGURE 7.4 Classes of biological safety cabinets. (A) Class I BSCs protect the environment by filtering contaminated air through a HEPA filter, but the cabinet does not provide a sterile environment within it. Class I BSCs are mainly used to house equipment that might generate aerosols but are not for use with sterile cultures. (B) Class II BSCs filter the environmental air through a HEPA filter before entering the cabinet to provide a sterile working surface. Environmental air is sucked into a grille at the opening of the cabinet to prevent it from contaminating the sterile working area. (C) Class III BSCs are completely sealed and air-tight, and all air entering or exiting the hood is filtered. Work in the cabinet must be performed by using the attached rubber gloves. (D) This researcher is performing cell culture in a class II BSC. Notice the grille at the front that prevents environmental air from entering the working area. The great majority of research and clinical laboratory work involving viruses is performed in a Class II BSC.

exposed. Therefore, there exist four **biosafety levels (BSLs)** that specify what precautions must be taken with different pathogens (Table 7.3). Some notable differences between the BSL levels are summarized below:

Biosafety level 1 (BSL1) is for work involving well-characterized agents not known to cause disease in healthy adult humans. These pathogens present minimal potential hazard to laboratory personnel and the environment. All material must be handled in an appropriate way and decontaminated after use, and workers must use gloves for protection, along with a lab coat and safety glasses, if warranted. Work with BSL1 agents does not require a BSC, unless cell cultures require the use of one to maintain sterility.

Biosafety level 2 (BSL2) is for work with agents that are known to pose moderate hazards to personnel and/or the environment. It includes all the precautions of BSL1 but also requires that laboratory personnel are supervised, receive specific training in handling the pathogenic agents, and conduct any work that may generate infectious aerosols or splashes in a BSC.

Biosafety level 3 (BSL3) is for work that could cause serious or potentially lethal disease through inhalation. BSL3 work includes all the precautions of BSL2 but also requires a special BSL3-level laboratory that

TABLE 7.3 Biosafety Levels Required for Work With Certain Viruses

Biosafety level	Examples of viruses worked with at this level
BSL1	Bacterial viruses
	plant viruses
	Nonhuman insect viruses
BSL2	Hepatitis A virus
	Hepatitis B virus
	Hepatitis C virus
	Hepatitis E virus
	Human herpesviruses
	Seasonal influenzas
	Poliovirus
	Hantaviruses (for potentially infected serum)
	Lymphocytic choriomeningitis virus
	Rabies virus
	Human immunodeficiency virus
	Severe acute respiratory syndrome–associated coronavirus (SARS-CoV)
BSL3	Hantavirus propagation
	1918 influenza virus
	Highly pathogenic avian influenza viruses
	Lymphocytic choriomeningitis virus strains lethal to nonhuman primates
	Human immunodeficiency virus (for large-scale volumes or concentrated virus)
	SARS-CoV propagation
	West Nile virus animal studies and infected cell cultures
	Eastern equine encephalitis virus
	Western equine encephalitis virus
	Venezuelan equine encephalitis virus
	Rift Valley fever virus
BSL4	Hendra virus
	Nipah virus
	Variola virus (smallpox)
	Crimean–Congo hemorrhagic fever virus
	Ebola virus
	Guanarito virus
	Junin virus
	Lassa virus
	Machupo virus
	Marburg virus

is entered through two self-closing doors and is under negative pressure so that contaminated air is drawn to another area and HEPA-filtered before leaving the room. Laboratory personnel must wear additional protective laboratory clothing, such as a gown, scrub suit, or coveralls, that is only worn while in the laboratory and then decontaminated or disposed of upon exit of the lab. Work must be performed in class II or class III BSCs, depending upon the virus.

Biosafety level 4 (BSL4) is for work with dangerous and exotic agents that pose a high individual risk of aerosol-transmitted laboratory infections and life-threatening disease that is frequently fatal and for which no vaccines or treatments are available. In addition to BSL3 standards, personnel must change out of their normal clothing and into laboratory clothing in a specific entry room. Some BSL4 labs use BSL3-protective laboratory clothing but class III BSCs are required for manipulating any infectious materials, which must be decontaminated when passing out of the BSC. Other BSL4 labs require the use of a one-piece protective plastic suit with a separate air supply to keep the suit under positive pressure, which would push out air if any cuts or tears were to occur (Fig. 7.5).

FIGURE 7.5 BSL4 protective suit. This researcher at the CDC is using a light box to count viral plaques in a BSL4 laboratory. Note that the suit is inflated because of the air supply, which keeps it under positive pressure in case of a leak in the suit. The researcher also must wear laboratory clothing under the suit (scrubs in this case). Upon exiting the lab, the worker will pass through a bleach shower to decontaminate the orange plastic suit, change out of his laboratory clothing, and then take a personal shower before changing back into his street clothes. *Photo taken by James Gathany, courtesy of the CDC and Dr. Scott Smith.*

This suit is decontaminated with a bleach shower upon leaving the lab. In either BSL4 lab scenario, workers must leave through a separate exit room, decontaminate their laboratory clothes, and shower before changing into street clothes. BSL4 labs must be in a separate building or clearly demarcated isolated zone within a building, have emergency power, be sealed from the environment, and provide multiple means of decontaminating the workspace and all infectious materials. These labs are reserved for the most dangerous infectious agents.

The majority of diagnostic cell cultures take place at BSL2. Generally, three to six cell lines are selected based upon the test being performed and cell cultures are inoculated with the clinical specimen. Although viruses cannot be seen under the light microscope, they biochemically affect the cells in which they are replicating, which sometimes leads to visible **cytopathic effects (CPEs)** that are distinguishable using a light microscope (Fig. 7.6A and B). For example, some viruses may cause cells that normally attach to the bottom of their culture vessel to round up and detach. Large bubblelike vacuoles are sometimes observed in the cytoplasm of infected cells, which may also swell or shrink, depending upon the virus. Other viruses cause adjacent cell membranes to fuse together, creating a **syncytium,** or giant multinucleated cell, that can have up to 100 nuclei within the cell. Some viruses cause lysis of the cells in which they replicate. Other viruses cause cell death or damage by hijacking the cell's transcription and translation machinery, leaving the cell at a deficit to translate its own proteins, including enzymes that are required for metabolic pathways.

Observation of CPEs—and how long it takes the virus to cause them—can provide clues for the diagnosis of the virus. For instance, adenoviruses cause cells to form grapelike clusters; herpesviruses cause cells to round up; and respiratory syncytial virus induces syncytia, as its name suggests. Other times, cell culture is used in conjunction with other assays. Electron microscopy can be used to identify the morphology of the virus, while **immunofluorescence assays (IFAs)** or **immunohistochemistry (IHC),** described in more detail below, can be used to definitely identify a specific virus within cells by recognizing viral antigens. For these assays, cells can be grown on slides or coverslips for easy removal from the culture.

Some types of clinical specimens bypass the need to infect cells in culture because they are infected cells or tissues taken directly from the patient. **Cytology** is the examination of cells, while **histology** refers to the examination of tissues. Blood, lung washings, and cerebral spinal fluid contain cells, and cells would also be collected during a cervical swab, which brushes some cells from the cervix and places them in a liquid preservative. The related Papanicolaou (pap) smear scrapes cells from the cervix but instead smears them across a slide that is then sent for analysis. A Tzanck smear uses a similar idea but is used to smear cells from skin lesions onto a slide for diagnosis of herpesvirus infections. These specimens would be subject to cytology. On the other hand, tissues are collected when a biopsy is taken. The tissue is sectioned into thin slices and undergoes histological examination at the diagnostic lab.

Most cells and tissue are devoid of color and therefore require colored stains for viewing under a microscope. Just as CPEs can be visualized in infected cell cultures, they can also be seen in cell or tissue specimens from a patient (Fig. 7.6C). Another type of CPE observed in infected cells or tissues is called an **inclusion body,** which is a visible site of viral replication or assembly within the nucleus or cytoplasm that can be observed with infection by some viruses (Fig. 7.6D; see also Fig. 13.9). Just as with infected cell cultures, cell or tissue specimens can be used for IFA or IHC.

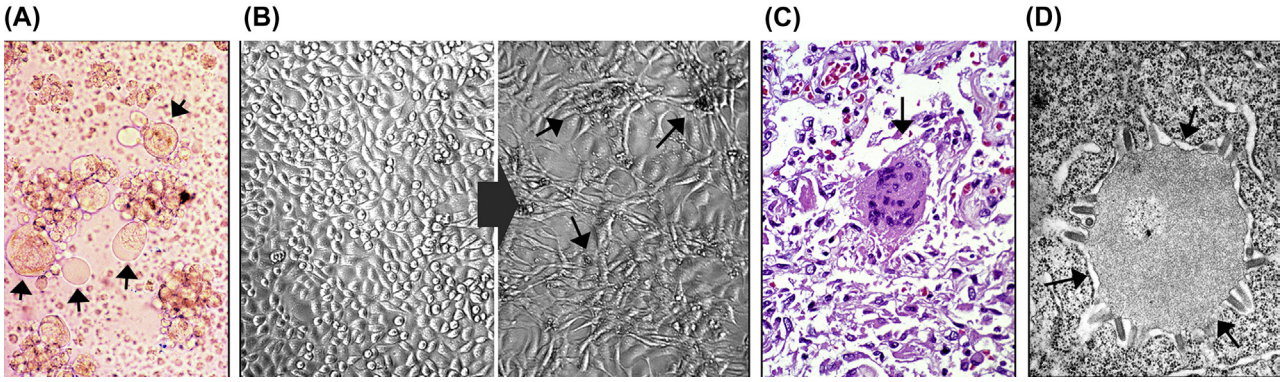

FIGURE 7.6 Cytopathic effects of viral infection. Viruses cause a variety of visible effects upon cells. (A) Human herpesvirus-6 infection causes ballooning of infected cells. *(Image courtesy of Zaki Salahuddin and the National Cancer Institute.)* (B) These photos show the cytopathic effects upon cells before (left) and after (right) infection with murine cytomegalovirus. Note the differences in morphology and organization of the cells. (C) A multinucleate syncytium caused by SARS-CoV in a histological section of stained human lung. *(Image courtesy of the CDC/Dr. Sherif Zaki.)* (D) A cytoplasmic inclusion body composed of viral proteins and nucleic acid is visible in this electron micrograph of a cell infected with Lagos bat virus. Note the bullet-shaped virions assembling from the inclusion body. *(Image courtesy of Dr. Fred Murphy and Sylvia Whitfield at the CDC.)*

7.3 DETECTION OF VIRAL ANTIGENS OR ANTIVIRAL ANTIBODIES

Although cell culture, cytology, and histology are valuable in providing visible clues as to the identity of a virus, some viruses cause similar CPEs or no visible CPEs at all. In these cases, it is necessary to use assays that detect viral antigens to prove the presence of a virus. This is accomplished by using antibodies that recognize specific viral antigens. Due to the advents of biotechnology, antibodies can be produced in large amounts and are commercially available. These antibodies recognize different antigens from a wide range of pathogens, including viruses. Several widely used and relatively fast assays make use of antibodies to identify the cause of a viral infection. "Immuno" is usually found in the name of the assay to indicate that it uses antibodies, which are produced by plasma cells of the immune system.

IFAs are performed on cells or tissues that have been affixed to slides and exposed to a fixative. The cells can be from patient specimens or they can be cell cultures that have been infected with patient samples. IFAs use antibodies that are conjugated to **fluorophores,** or fluorescent dyes, that give off a certain color when they are excited by a particular wavelength of light. The best-known fluorescent dye is **fluorescein isothiocyanate**, better known as **FITC**, which is excited by light of 490 nm (blue) and gives off light in the 519 nm range (green). In an IFA, FITC-conjugated antibodies specific for a certain virus are added in a liquid buffer onto the cells or tissue on the slide (Fig. 7.7A). If the viral antigen that is recognized by the antibody is present on the cell surface, perhaps because the virus was assembling at the plasma membrane or was in the process of infecting cells when the section was fixed, then the antibodies will bind to the viral antigen present. Alternatively, the cells can be permeabilized with a detergent to allow the antibodies to enter inside the plasma membrane and bind viral antigens there (Fig. 7.7B). In either case, the antibodies will not bind if the specific antigen is not present. After a sufficient period of time to allow binding of the FITC-labeled antibodies, the slides are washed with a buffer. Any antibody that is bound will remain bound, whereas unbound antibody will be rinsed off. The slides are examined under a fluorescence microscope, which contains a special lightbulb that can provide the wavelengths of light able to excite the fluorophores and filters that allow the viewer to see one emitted color at a time. In this case, if any green cells are seen, then the antibodies bound and the virus was present.

Because the antibody was directly bound to the specific antibody, this IFA is known as a **direct IFA,** or **direct fluorescent antibody** staining. However, sometimes conjugated antibodies are not available; in these cases, a second antibody must be used that is fluorescently conjugated and recognizes the primary antibody (Fig. 7.7C). This is a way to get around not having a conjugated primary antibody. It can also be used to amplify a weak signal when not much viral antigen is present. Because this assay requires a secondary antibody and the fluorochrome is not attached to the primary antibody, it is known as an **indirect IFA.** IFA is used for the identification of a variety of viruses, including several different herpesviruses, influenza, measles, mumps, and adenovirus (Fig. 7.7D).

IHC relies upon the same principles as an IFA except that the antibody is conjugated to an enzyme instead of a fluorescent molecule. After the tissue is exposed to the enzyme-bound primary antibodies, a liquid substrate is added to the slide. If the enzyme-linked antibodies have bound to the tissue, the enzyme will cleave the substrate and a visible colored precipitate will be deposited on the slide. IHC is visible using a normal light microscope and does not require the use of a fluorescence microscope. IFAs or IHC can take as little as a few hours to perform.

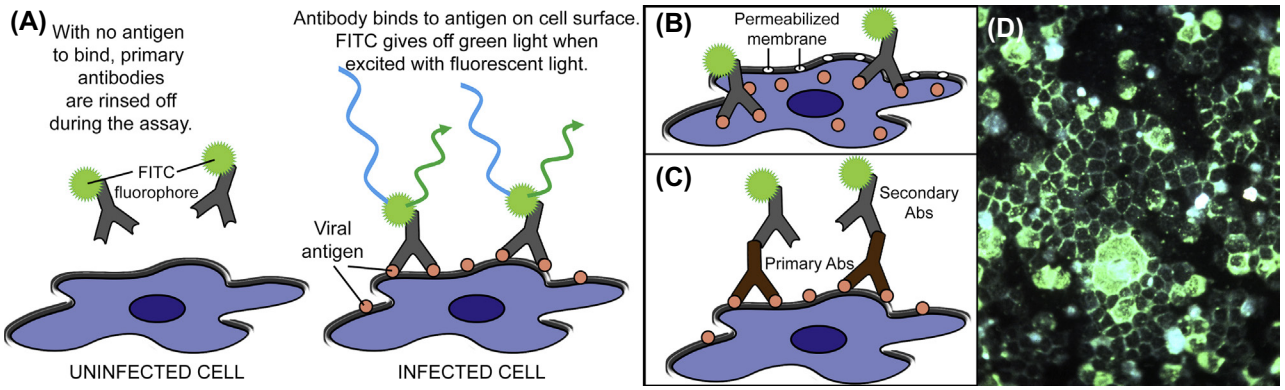

FIGURE 7.7 Immunofluorescence. Immunofluorescence uses virus-specific antibodies to verify infection. (A) A buffer containing FITC-conjugated antibodies is added to fixed cells or tissues (from cell culture or cell/tissue specimens). The antibody binds to cognate viral antigens expressed on the surface of infected cells, which prevents it from being rinsed off the slide after incubation. The "stained" cells are examined under a fluorescence microscope to excite the FITC dye, which fluoresces green. This process can also be used to detect intracellular antigen by permeabilizing the plasma membrane to allow the antibodies to enter the cell (B). Some antibodies are not commercially available as already conjugated, so a FITC-labeled secondary antibody that recognizes the first antibody must be used (C). (D) is an example of an IFA performed on cells to verify the presence of respiratory syncytial virus. The green cells are therefore infected. *Photo courtesy of the CDC and Dr. Craig Lyerla.*

An extension of this concept takes place in an **enzyme immunoassay (EIA)**, also known as an **enzyme-linked immunosorbent assay (ELISA)**. Like IFA or IHC, EIAs/ELISAs also detect viral antigens using antigen-specific antibodies. Unlike IFAs or IHC, however, they do not use cells or tissues. Instead, they assay for viral antigens in liquid samples, such as serum or urine. A **sandwich ELISA**, starts by adding antibodies in a buffer to a special plastic plate that has been treated to bind proteins—including antibodies—to it (Fig. 7.8). These are known as "capture antibodies" because they will be used to capture the antigen from the patient sample. Once the antibodies have bound, the wells of the plate—usually there are 96 of them—are rinsed with a buffer to remove any unbound antibody. The plate is **blocked,** meaning that a buffer is added that contains nonspecific proteins, which attach to the plastic wells wherever there is no antibody bound. The wells are rinsed again, and then the patient's sample is added. If the antigen is present in the sample, either because the virus or pieces of the virus are present, then the antigen will bind to the capture antibody. In contrast, if no viral antigen is present in the patient sample, then the capture antibodies will not capture any antigen.

The wells are again rinsed to remove any leftover patient sample. Next, an antibody is added that is conjugated to an enzyme, just as with IHC. (This is where the "enzyme-linked" part of the ELISA name is derived; the "immunosorbent" part indicates that antibodies, the "immuno" part, absorb the antigen.) The enzyme-linked antibody, called the detection antibody, also specifically recognizes the antigen and will create a "sandwich" with the antibodies as the bread and the antigen as the meat—hence the name "sandwich ELISA." After sufficient time for binding to occur, the wells are again rinsed. The detection antibody will remain bound to the plate if the antigen was present, but if no antigen has bound the capture antibody, then there will be nothing for the detection antibody to bind and it will be rinsed out of the well.

The final step is to add the liquid enzyme substrate. If the detection antibodies are present—meaning that the capture antibody bound antigen because the antigen was present in the patient sample—then the enzyme attached to the detection antibodies will cleave the substrate, producing a visible color in the well. If no antigen was present, then there will be no detection antibody to catalyze the substrate reaction. No color will occur.

Spectrophotometers measure the intensity of light, including colored light. A special 96-well spectrophotometer (Fig. 7.9B) measures the color in each well and provides an optical density value that indicates the relative amount of antigen present in each well. If a standard set of samples with known concentration is also tested in the same ELISA plate, then the value obtained with the patient sample can be compared to the standards to obtain a quantitative value. Positive and negative controls are always performed in each ELISA to verify that the assay was performed correctly.

Each step of an ELISA usually allows around an hour or two for binding to occur. Therefore, an ELISA takes several hours to perform all together, but results are usually available the same day the clinical specimen was received. Testing for the hepatitis B surface antigen is performed using a sandwich ELISA.

ELISAs can be used to not only detect viral antigens, but to determine the presence of antibodies against a virus, known as **antiviral antibodies.** Assaying for antiviral antibodies is an indirect way of seeing if a patient has been exposed to a virus, and ELISAs that differentiate between IgM and IgG can provide clues as to whether a patient has been newly exposed. If a person has IgM and becomes positive for IgG over the course of days, then the person is experiencing a primary infection. If the person has high levels of IgG, then it is a secondary or recurrent infection. Antibody isotypes are also used to diagnose congenital infections. IgG crosses the placenta from the mother to the child and is therefore not helpful in diagnosis, but IgM is too large to cross the placenta. Therefore, if IgM is present in the infant's blood, it is made by the infant's immune system and indicates an infection in the child.

To assay for antibodies, the viral antigen is coated onto the bottom of the ELISA plate (Fig. 7.8). The patient sample is added, and if the patient possesses antibodies that recognize the viral antigen, then the antibodies will attach to the antigen on the plate, thereby immobilizing the antibody to the plate, as well. As with a sandwich ELISA, the next step is the addition of an enzyme-linked detection antibody. In this case, the detection antibody recognizes the patient's antibodies. Finally, the wells are rinsed and a substrate is added. If the patient possesses antibodies against the viral antigen, the patient's antibodies and subsequent detection antibodies would have bound to the antigen coated in the wells, and the presence of the enzyme would cleave the substrate, producing color (Fig. 7.9).

A **western blot** utilizes a similar process, except that the protein antigens are immobilized in a gel first. In the same way that DNA can be separated using agarose gel electrophoresis, proteins can be separated by size using polyacrylamide gel electrophoresis (PAGE)(Fig. 7.10A). The proteins embedded into the polyacrylamide gel are transferred onto a membrane made of nitrocellulose. At this point, the membrane functions like the wells of the ELISA plate. Enzyme-linked antibodies against the proteins that have been separated are added in a liquid buffer to the membrane and will bind specifically to the bands of protein antigens (Fig. 7.10B). A substrate is added, and the enzymes present produce detectable color, light, or fluorescence, depending upon the enzyme and substrate used (Fig. 7.10C).

Western blots are often used to confirm positive ELISA results. For example, if a person takes an oral HIV test (that uses saliva) and receives a positive result, the test must be confirmed using a western blot. In this scenario, HIV antigens are separated through PAGE, transferred to

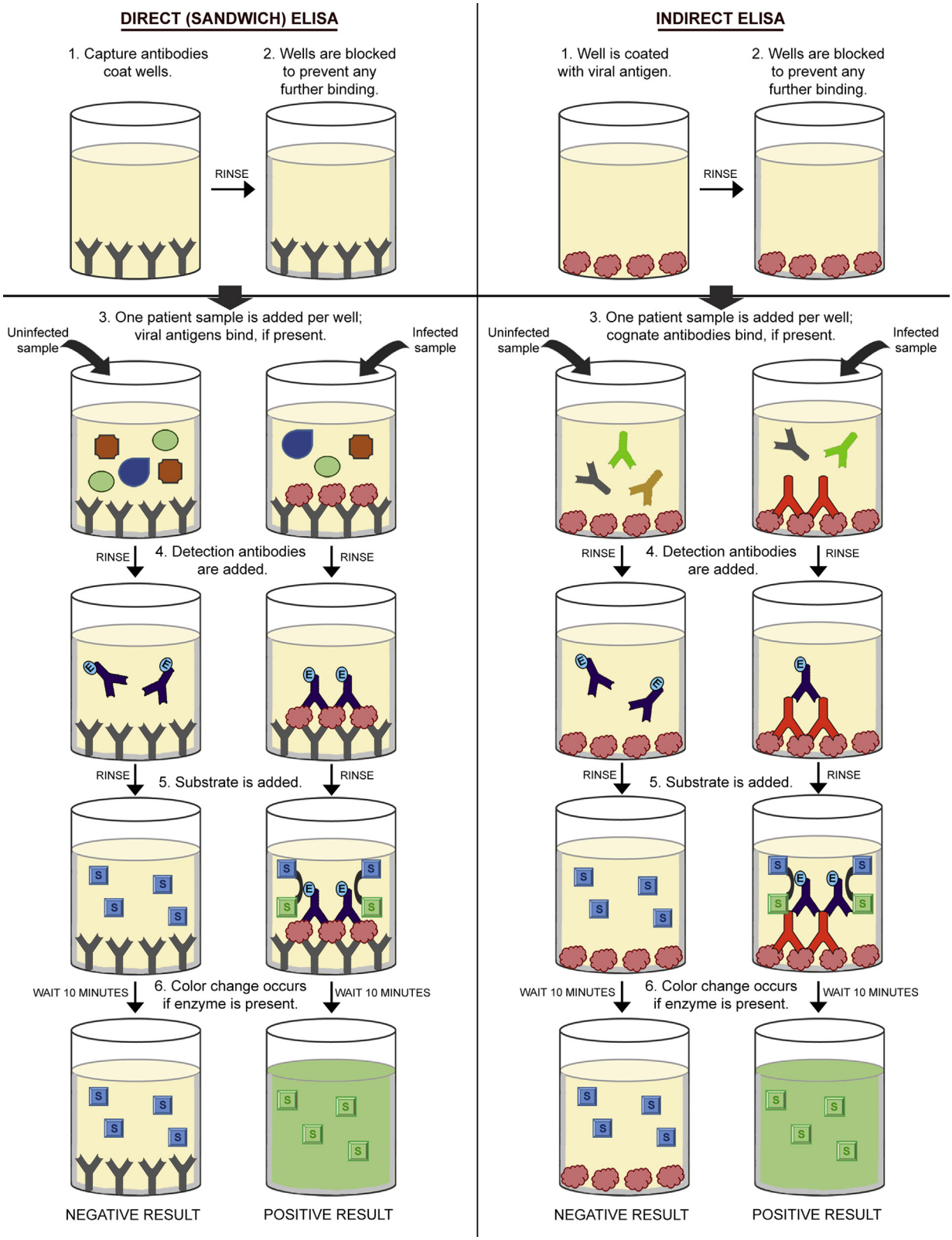

FIGURE 7.8 Enzyme-linked immunosorbent assay (ELISA). An ELISA measures viral antigens (direct "sandwich" ELISA) or antiviral antibodies (indirect ELISA). In a sandwich ELISA (left), capture antibodies specific for the viral antigen are coated on the bottom of the wells, which are then blocked to prevent nonspecific binding. The wells are rinsed out in between each step to remove unbound molecules. The patient samples are added, one sample per well, at which point any cognate viral antigens will be bound by the capture antibodies. Next, enzyme-linked detection antibodies are added to each well and also bind the viral antigen, if present. When the substrate is added, it will be cleaved by the enzyme, if present, to form a visible color that is read by a spectrophotometer. In an indirect ELISA (right), the viral antigen is coated to the bottom of the wells and blocked. When the patient samples are added, any patient antibodies that recognize the viral antigen will bind to the plate, as well. Secondary enzyme-linked antibodies are added that recognize human antibodies, and the enzyme cleaves the substrate when added to produce color.

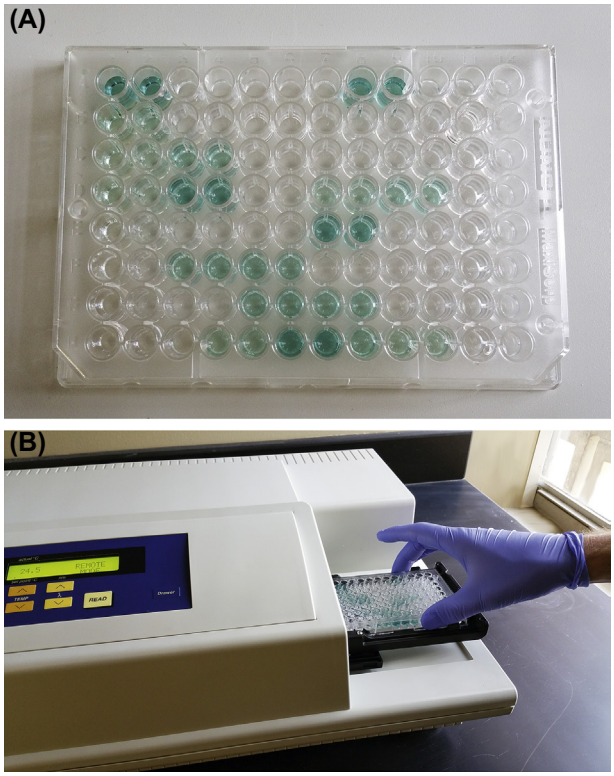

FIGURE 7.9 ELISA. (A) Note the variations in the color in the wells of the 96-well ELISA plate. A darker color indicates a higher amount of antigen or antibody was present in the patient sample. (B) An ELISA about to be read in a spectrophotometer.

a nitrocellulose membrane, and exposed to the patient's serum. If the patient has anti-HIV antibodies, they will bind onto the membrane where the HIV antigens are found. A secondary, enzyme-linked antibody will provide the enzyme that will cause a colored band to be produced when exposed to a substrate. (Western blots are still recommended for confirmation of oral HIV tests, although nucleic acid testing is now the choice of confirmatory test for positive HIV blood tests.)

Agglutination reactions take place when the binding of antibodies to antigen causes a visible clumping, or agglutination. Latex agglutination tests use the same principles as ELISAs or western blots, except the antigen or antibody is bound onto small latex beads (Fig. 7.11A). To test for patient antibodies, the viral antigen is coated onto the latex beads. When mixed with patient serum, the antibodies will bind to the antigen-coated latex beads. Because antibodies have two antigen-binding sites, each arm of the antibody is able to bind a different bead. The result is that the beads are splayed out in a lattice formation—the beads have agglutinated, and a visible "clump" has formed. If the patient does not have antibody against the particular viral antigen, then the latex beads will not agglutinate. Agglutination reactions can take place in tubes, 96-well plates, on slides, or using cardboard cards. Like ELISAs, latex agglutination tests can

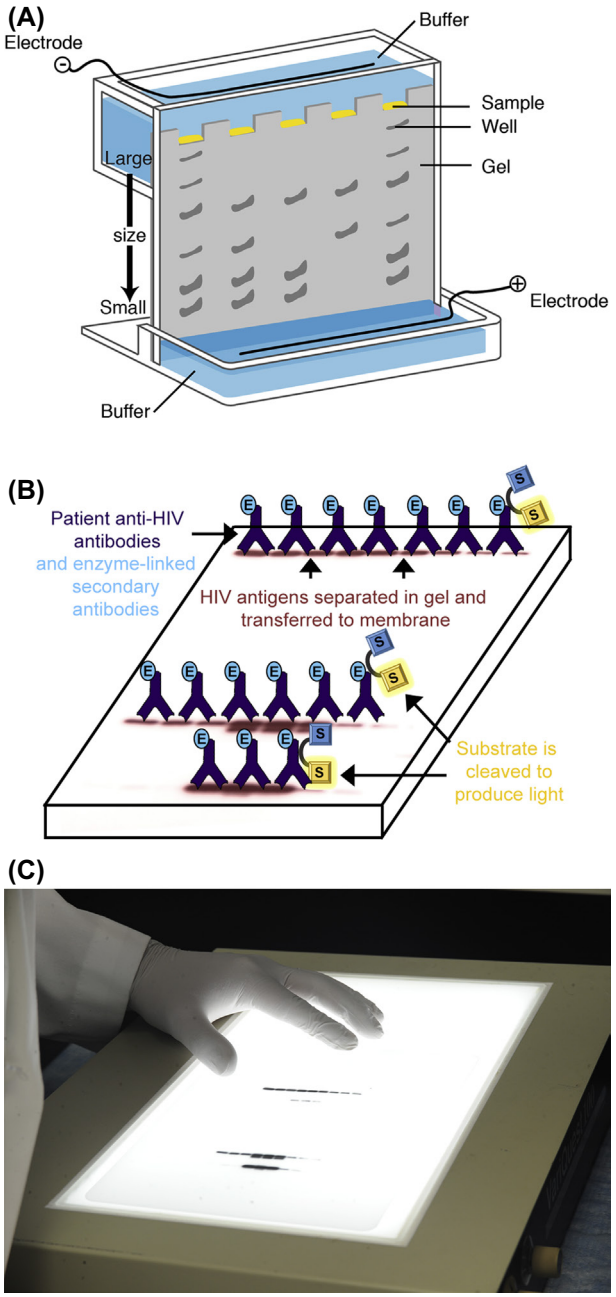

FIGURE 7.10 Western blot. Western blots work on the same principles as an ELISA, except the proteins are separated in a gel and transferred to a membrane instead of using wells. (A) To confirm a positive HIV result, a variety of different HIV antigens are separated using PAGE. The samples are loaded in the wells at the top of the gel, a charge is applied to the gel, and the proteins separate by size, with the smallest proteins traveling farthest in the gel. (B) Following PAGE, the HIV proteins are transferred to a nitrocellulose membrane. The patient serum sample is incubated with the membrane, and if the patient has anti-HIV antibodies, they will bind to the HIV proteins on the membrane. An enzyme-linked secondary antibody is added that recognizes human antibodies (just the enzyme part is shown here to save room). A substrate is then added that produces a detectable signal; in this case, the substrate gives off light that will create a dark mark when placed against X-ray film (C). In this scenario, the patient is positive for anti-HIV antibodies and thus has been infected with HIV. *Illustration in (A) by Darryl Leja and photo in (C) taken by Maggie Bartlett, National Human Genome Research Institute.*

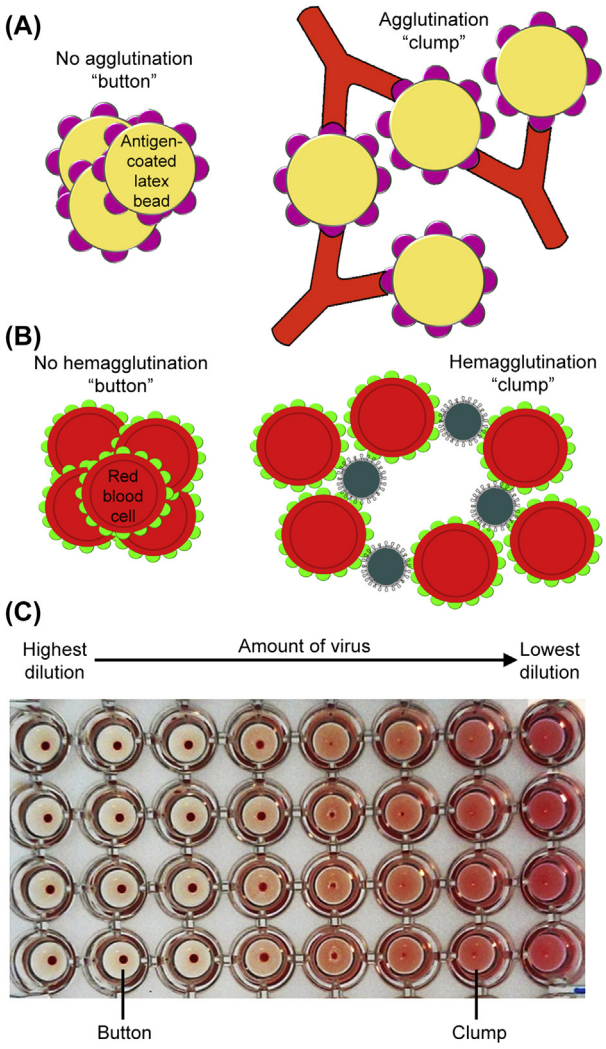

FIGURE 7.11 **Agglutination/hemagglutination.** (A) Latex agglutination assays coat viral antigen onto latex beads. If antibodies are present that recognize the antigen, they will agglutinate the beads. The same procedure can be performed to assay for the presence of viral antigens by coating antibodies on the beads. (B) Hemagglutination refers to the agglutination of red blood cells specifically. Several viruses, including influenza virus and measles virus, possess hemagglutinin proteins that bind to red blood cell surface glycoproteins. Hemagglutination can be used to identify these viruses. (C) The results of a hemagglutination reaction that show hemagglutination occurring as increasing amounts of virus are titrated into the wells. The "button" on the left and the "clump" on the right show the visible change that occurs with hemagglutinated blood (on right). *Image courtesy of Liu, Y., et al., 2013. Poly-LacNAc as an Age-Specific Ligand for Rotavirus P[11] in Neonates and Infants. PLoS One 8(11), e78113.*

also test for viral antigen in a patient sample by coating antibodies onto the beads that recognize the antigen. Several antibody-coated beads will bind to one antigen, resulting in visible agglutination.

Hemagglutination refers to the agglutination of red blood cells. A handful of viruses, including influenza virus, measles virus, mumps virus, rubella virus, and rabies virus, cause the hemagglutination of red blood cells. The viral attachment glycoproteins (aptly named "hemagglutinin" in influenza, measles, and mumps viruses) bind to the surface of red blood cells and agglutinate them, similarly to how antibodies do (Fig. 7.11B and C). Hemagglutination assays have been used to show the presence of these viruses in samples. A similar assay tests for the antibody levels in a patient sample against one of the viruses. A fixed amount of virus is added to each well, and then the patient serum is titrated into the wells at different dilutions. If the patient has antibodies against the virus, the antibodies will bind the virus and prevent it from hemagglutinating the red blood cells. By using dilutions of the patient serum, the relative amount of antibody can be determined by noting which dilutions do or do not prevent hemagglutination.

An assay that uses antibodies in a similar fashion as to the assays described above is called the **lateral flow immunoassay (LFIA)**. In this case, the presence of a virus (or antibodies against a virus, if the test assays for antiviral antibodies) will result in a colored band appearing in a particular window on the test. Pregnancy tests are the best-known LFIAs, but LFIAs are available that test for HIV antibodies, dengue virus antibodies, rotavirus, respiratory syncytial virus, and influenza virus. They can be in the form of a dipstick, like a pregnancy test, or they can be a small plastic test that requires a drop of sample be placed into a sample well (Fig. 7.12A).

In the LFIA, the test reagents are added onto a nitrocellulose membrane that draws the liquid along the length of the strip using capillary action (Fig. 7.12B). The test strip contains three major zones: a reaction zone, test zone, and control zone. In the case of the influenza LFIA, the patient specimen (usually a nasal or nasopharyngeal swab) is mixed with a buffer that disrupts any virions that are present, releasing the viral antigens. A drop of the liquid sample is placed in the sample well on one end of the LFIA test, and the viral antigens begin to move through the membrane through capillary action to the reaction zone of the test. The reaction zone contains free antibodies that are bound to gold beads or blue latex beads, which will be the basis for the formation of color later in the test. As the viral antigens pass through the reaction zone, the bead-linked antibodies bind to the antigens, and the antigen–bead complex continues moving through the membrane. The test zone is the next zone encountered. In the test zone, other antibodies are immobilized to the membrane and recognize the viral antigens that are bound to the reaction zone bead–linked antibodies. The binding of the antigen–bead complex to the test zone antibodies immobilizes the antigen–bead complexes to the test zone area, creating an antigen sandwich. As the beads begin accumulating, the color of the

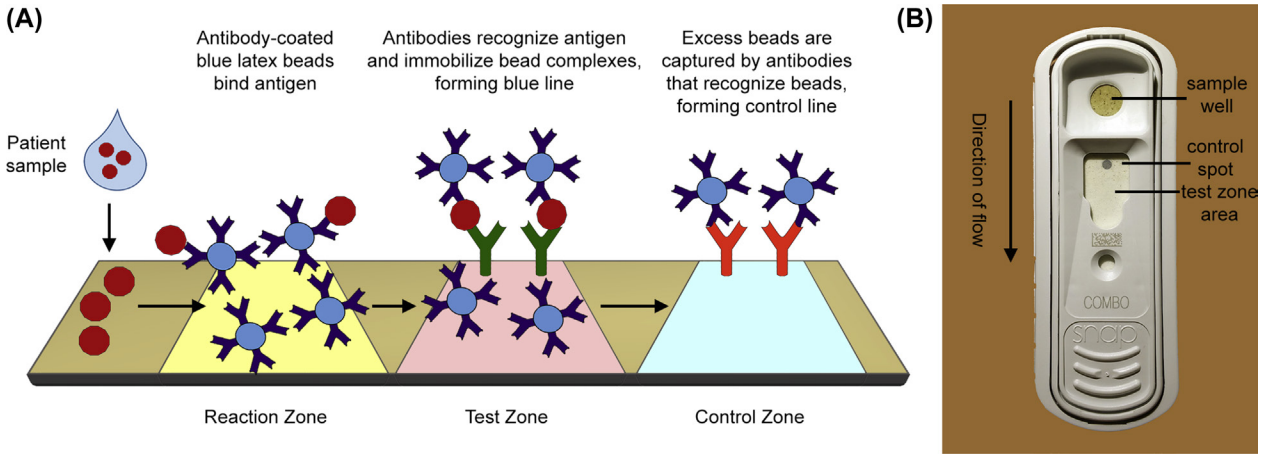

FIGURE 7.12 Lateral flow immunoassay (LFIA). (A) Lateral flow immunoassays have three major zones: a reaction zone, a test zone, and a control zone. The patient's sample is added to the sample well of the assay and begins moving though the membrane via capillary action. In the reaction zone, antigens found in the patient's sample bind to free antibody-coated beads. The bead-antigen complexes continue traveling to the test zone, where membrane-bound antibodies bind to the antigen, thereby immobilizing the beads. As the beads accumulate, a colored line is formed. Excess bead complexes continue moving through the membrane and are eventually bound by control zone antibodies that recognize the bead complex, rather than antigen. As excess beads accumulate here, a colored control line is formed. (B) LFIAs are useful for diagnosis of viral infection in animals, as well. This LFIA tests for two feline viruses, feline immunodeficiency virus and feline leukemia virus. Note that the control spot is present, but there are no other colored spots in the test zone area, indicating that the animal was negative for both viruses.

beads becomes apparent. Blue latex beads provide a blue band, and gold beads appear as a red band. This indicates a positive result—that the person's specimen contained the virus.

The final zone, the control zone, contains antibodies that will bind the bead complex. If the reaction zone beads are not bound to antigen, they will continue flowing through the test zone and be captured by the control zone antibodies. In fact, there are many more reaction zone beads than antigen, so some beads will be free even in the presence of antigen and make it to the control zone. The capture of the beads in the control zone produces a visible line in this section of the test. This is the positive control to show that the test worked.

The HIV or dengue virus LFIAs use the same principles, except they assay for the presence of antibodies against the viruses, rather than detecting the viruses themselves.

7.4 DETECTION OF VIRAL NUCLEIC ACIDS

Nucleic acid testing (NAT) has replaced many of the traditional, slower assays in diagnostic labs. Detecting viral nucleic acids is a sensitive and specific way to screen for viruses within a patient sample, and new methods allow for the screening of many viruses simultaneously. Additionally, NAT is able to detect the presence of viruses for which no other test currently exists. NAT results are available the same day the sample is processed.

NAT assays rely upon the principle of **polymerase chain reaction (PCR)**, which recapitulates the process of DNA replication in the laboratory by providing the molecules necessary to copy DNA (see Chapter 3, Features of Host Cells: Cellular and Molecular Biology Review for a review). In the process of PCR, DNA (including any viral DNA present) is isolated from the clinical specimen, generally blood cells or tissue, and added to a tube containing primers, DNA polymerase, and nucleotides (Fig. 7.14). The tube is placed in a **thermocycler,** a bench-top machine that simply changes the temperature of the tube, as its name suggests. In the *denaturation* stage of PCR, the thermocycler heats the DNA, usually to 95°C, which breaks the hydrogen bonds holding the two DNA strands together and so they separate from each other. In the *annealing* stage, the temperature is reduced to allow the binding (annealing) of two **primers** to the separated DNA. The primers are complementary to the sequence to which they bind, and they flank the region to be amplified, one primer on each strand. The annealing temperature is determined by the composition of the nucleotides in the primers, but is usually around 55°C.

In the final stage, *extension*, the thermocycler raises the temperature to the optimal temperature for the DNA polymerase enzyme. In this case, a special polymerase from the bacterium *Thermus aquaticus*, called Taq polymerase, is used. *Thermus aquaticus* was discovered to live in the hot springs of Yellowstone National Park, and its DNA polymerase is evolved to withstand high temperatures, such as those found in the hot springs. Taq polymerase is used for PCR because a human DNA polymerase would be denatured by the high temperatures required for the denaturation stage. The thermocycler holds the temperature at 72°C, and Taq polymerase extends

In-Depth Look: Counting Viral Particles Using a Plaque Assay

It is often necessary to know how many infectious virions are present in a sample. The diagnostic techniques described in this chapter identify the presence of a virus in a sample, or even the amount of viral nucleic acid, but these assays cannot determine the amount of virus present that is capable of productively infecting cells. A very common virology technique to determine this is known as the **plaque assay**, which measures the number of virions in a sample that are able to initiate infection of target cells.

Microbiologists measure viable bacteria by determining the number of individual bacterial cells in a sample, each of which will form a distinct bacterial colony. This results in the number of colony-forming units, or CFUs, in a sample. Plaque assays measure the number of individual cells that were infected by a single virion, each of which forms a **plaque**, or clearing, as the virus spreads among neighboring cells. This results in the number of **plaque-forming units (PFUs)** in a sample, the indication of viral infectivity.

To perform a plaque assay, the first step is to use cell culture to plate cells into several cell culture dishes or a multiwell plate—6-well or 24-well plates are often used for this purpose—in a liquid medium to support their growth (Fig. 7.13). The cell line used must be permissive to infection with the virus that is being studied. The cells are allowed to grow to near **confluency,** meaning that they have grown to completely cover the bottom of the cell culture vessel. At this point, the medium is removed from the wells. Tenfold serial dilutions of the initial sample are made, in case the initial sample has too much infectious virus and ends up harming the entire well of cells, and 0.1 mL of the experimental samples are added to individual wells. The plate of cells is gently shaken on a flat surface, moving the plate in the motion of a "plus" sign, to ensure that the virus that was just added is equally distributed over all the cells.

The virus is given time to bind and enter the cells (<1 h), at which point the cells are covered with a layer of cooled agarose that has been mixed with medium. Having entered random cells in the well, the virus will replicate over the course of the following days, and the agarose ensures that released virions are only able to infect cells immediately adjacent to the infected cell.

As the virus replicates and infects more cells, it begins forming plaques, or clearings, that are caused by the cytopathic effects upon the cluster of infected cells. In some cases, the virus is lysing infected cells, and in other cases, the virus has interfered with enough cellular processes to cause damage or death to the infected cells. Eukaryotic viruses typically take 3–5 days to form plaques that are large enough to see. Because cells are clear, a dye is used to stain the living cells, which leaves the clear plaques visible when the well is inspected.

The number of plaques are counted, giving the number of PFU per well. However, this needs to be converted into PFU/**mL** in the original sample, so the number of plaques must be divided by the dilution of the sample and by 0.1 mL, since that was the amount added to the cells. Therefore, 0.1 mL of a 0.001 dilution (1/1000) that resulted in 54 plaques means that the starting sample had 540,000 PFU/mL in the initial sample—54/0.1 mL/0.001 = 540,000. It is best to write this using scientific notation: the amount of infectious virus in the starting sample was 5.4×10^5 PFU/mL.

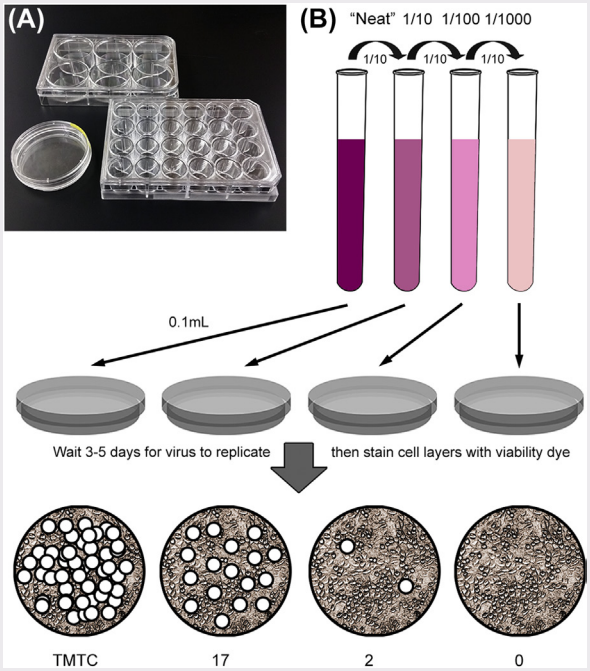

FIGURE 7.13 A plaque assay determines the amount of infectious virus in a sample. (A) Plaque assays are performed in a variety of different types of culture vessels, depending upon the size of the plaques that are formed. Shown here are a tissue culture dish, 6-well plate, and 24-well plate. (B) To perform a plaque assay, the undiluted "neat" sample is diluted several times using 10-fold dilutions. 0.1 mL of each dilution is added to an individual dish of cells. After allowing for time for the virus to enter the cells (about 1 h), a layer of agarose is used to overlay the cells to ensure lateral infection by infected cells. Depending on the virus, it generally takes 3–5 days for the visible sites of replication, called plaques, to be visible. At this point, the live cells are stained, and the clearings are counted (see Fig. 7.5). A dilution that has multiple but distinct plaques should be chosen to determine the PFU/mL in the original sample. In this case, the undiluted sample had 1.7×10^3 PFU/mL.

the 3′-end of the primer using the available nucleotides, creating a double-stranded piece of DNA from the single-stranded template. In this way, one double-stranded piece of DNA was separated and replicated to create two copies.

The stages of PCR are repeated, usually 30–35 times, to create billions of copies of the target DNA segment. In the case of viral diagnosis, the primers would be specific for a piece of the viral genome. Good primers will bind to just the

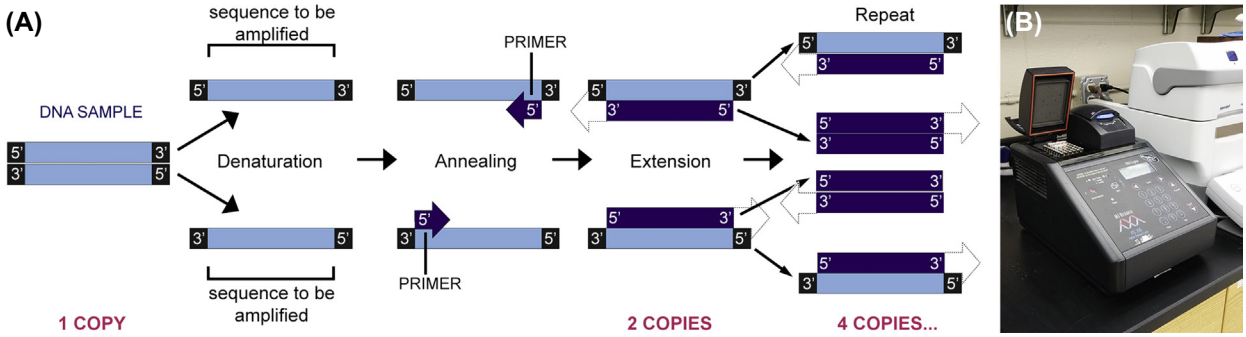

FIGURE 7.14 Polymerase chain reaction (PCR). PCR amplifies a specific sequence of DNA, based upon the location of primers. (A) In *denaturation*, high heat separates the two strands of the DNA sample. In *annealing*, the temperature is lowered, which allows the primers to hybridize to the strands. In *extension*, DNA polymerase extends the primers, creating the new strand. One DNA strand has now been copied into two. The three stages are repeated 30–35 times to generate billions of copies of the target sequence. If amplicons are created, then it means viral nucleic acid was present in the patient's DNA sample. (B) A thermocycler, the machine that controls the reaction temperature during PCR. (The white machine on the right is another brand of thermocycler.)

viral DNA being amplified and not to any isolated cellular DNA or genomes of other viruses.

What about viruses with an RNA genome? In this case, **reverse transcriptase PCR (RT-PCR)** is carried out. It involves the same steps as those performed with PCR, but because the viral genome is RNA, it must be reverse transcribed into cDNA first. This is accomplished using reverse transcriptase, which transcribes cDNA from an RNA genome. The reverse transcriptase used in RT-PCR is derived from retroviruses, generally Moloney murine leukemia virus or avian myeloblastosis virus. This additional step creates cDNA that is then amplified using PCR as above.

The amplified DNA fragment, known as an **amplicon,** can be detected in several different ways, but agarose gel electrophoresis remains the simplest and cheapest method. Agarose gel electrophoresis uses electricity to separate DNA fragments in an agarose gel. The distance traveled by the DNA is based upon the fragment size, with smaller fragments traveling farther in the gel than larger fragments. After separation is complete, the gel is stained with a chemical known as ethidium bromide that intercalates in between the base pairs of the DNA and fluoresces when exposed to UV light. A fluorescent band, therefore, indicates that DNA is present and was amplified by the PCR reaction. Because smaller fragments travel farther through the gel, the relative location of the band can verify the band that was produced is the anticipated size when compared to a known DNA ladder.

Multiplex PCR allows for the amplification of several different pieces of DNA at the same time in the same tube. This technique involves the same process as normal PCR or RT-PCR, but multiple primer pairs are added to the reaction tube. Each primer pair is designed to recognize the nucleic acid from a distinct virus, and the size of each amplicon produced is different for each virus. This allows for the simultaneous amplification of DNA from several viruses at one time, and analyzing the size of the amplicon reveals which viruses were present in the initial starting sample.

PCR was invented by Kary Mullis in 1983, and this molecular biology technique has been built upon and adapted to produce numerous assays of great utility. A modification of PCR that is ubiquitously used is known as **real-time PCR**, so named because the user can monitor the amplicon amplification in real time using a special thermocycler. A great advantage of monitoring the reaction as it proceeds is that the rate of amplification can be noted. Traditional PCR machines analyze the product after the completion of all cycles, which makes it impossible to know if the reaction had plateaued at an earlier cycle because of limitations in reagents, such as nucleotides. Real-time PCR machines address this concern by providing a visual graph of the amplification. An advantage of being able to see the amplification in real time is that the quantity of the PCR product can be used to back calculate the amount of starting template nucleic acid that was present, since we know that each cycle doubles the amount of DNA. For this reason, real-time PCR is also known as **quantitative PCR.** Therefore, the standard abbreviation for real-time PCR, is **qPCR.** (Recall that RT-PCR stands for reverse transcriptase PCR, *not* real-time PCR!) RT-PCR can also be performed using qPCR; this technique is known as RT-qPCR.

How is DNA amplification monitored in real time during qPCR? There are two major methods, and both involve

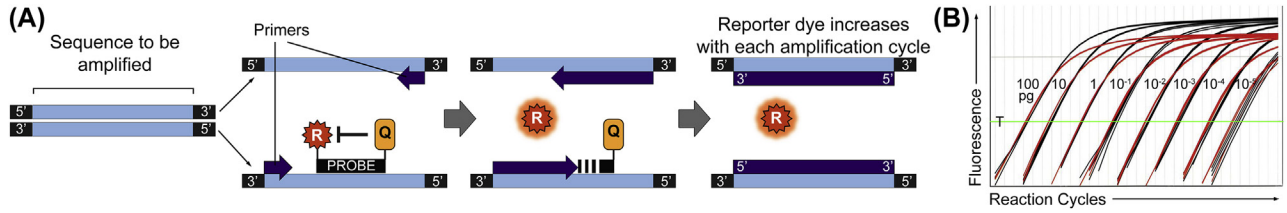

FIGURE 7.15 Real-time PCR (qPCR). Real-time PCR works on the same principles as PCR, except that it measures the fluorescence of a reporter dye in real time to monitor the amplification reaction. DNA-binding dyes are used, as are probes attached to a reporter dye and quencher molecule (A). The probe hybridizes to a section of the target DNA in between the two primers. As long as the reporter and quencher are physically close, the quencher absorbs any fluorescence and none is given off. As the primer is extended by DNA polymerase, the probe is broken down, releasing the reporter dye and quencher. Free from the quencher, the reporter dye fluoresces. The fluorescence increases as each cycle is repeated. (B) A real-time PCR amplification plot showing the amplification of eight different concentrations of JC polyomavirus (JCPyV) DNA. The number of reaction cycles is on the X axis, while the fluorescence generated in the reactions is on the Y axis. A few important things to note: The samples that start with more DNA are amplified more quickly (require fewer cycles to reach a set threshold, T). Also, each of the cycles eventually plateaus around the same fluorescence, even though we know some samples contain more DNA than others. This is why the samples are compared at the threshold limit (green line). This was multiplex qPCR: black lines are the amplification of a JCPyV protein–coding genomic sequence, while the red lines are the amplification of a noncoding genomic region. *Amplification plot reprinted from Ryschkewitsch, C.F., et al., 2013. Multiplex qPCR assay for ultra sensitive detection of JCV DNA with simultaneous identification of genotypes that discriminates non-virulent from virulent variants. J. Clin. Virol. 57(3), 243–248, Copyright 2013 with permission from Elsevier.*

the use of fluorescence. In the first method, a fluorescent dye is used that intercalates into double-stranded DNA, in the same way that ethidium bromide does. An example of a fluorescent DNA-binding dye is SYBR Green, which is excited with light of 488 nm and emits light at 522 nm (green). The real-time thermocycler is able to provide the excitation wavelength and has detectors to measure the emitted wavelength. Another method involves fluorescent reporter probes, such as Taq-Man® probes (Fig. 7.15A). Like a primer, the probe recognizes a sequence of the DNA target, but it is located in the middle of the amplified sequence, in between the primers. Attached to the probe are a fluorescent **reporter dye** at one end and a **quencher** at the other. As long as the reporter dye and quencher are attached to the probe, the quencher absorbs the fluorescence emitted by the excited reporter dye (through the process of fluorescence resonance energy transfer, or FRET). Taq polymerase breaks down the nucleotides of the probe as it amplifies the DNA to which the probe is bound, releasing the reporter dye and quencher from the probe. When this occurs, the two are physically separated and the quencher molecule can no longer inhibit the reporter's fluorescence. As with the fluorescent double-stranded DNA dyes, reporter dyes are excited and detected by the thermocycler.

Using either method, an increase in fluorescence indicates an increase in amplified product. A **DNA amplification plot** is created as the measurements of fluorescence are taken in real time (Fig. 7.15B). This allows the DNA product to be quantified at a cycle number where the amplification is in exponential phase, which can be used to back calculate the amount of starting template DNA. Multiplex qPCR assays have also been developed that allow for the simultaneous detection of several fluorescent probes in one tube, one for each amplified viral DNA segment.

PCR and qPCR amplify specific sequences of viral DNA. On the other hand, **DNA microarrays** rely upon the **hybridization** of viral nucleic acid segments to a synthesized piece of DNA. "Hybridization" refers to the complementary binding of two nucleic acid pieces to each other; for instance, primers hybridize with their target DNA during PCR. With DNA microarrays, thousands of short single-stranded pieces of DNA, called **oligonucleotides (oligos),** are immobilized onto a small silicon chip or glass slide (Fig. 7.16B). Oligos, like primers, are able to be synthesized in the laboratory using a specialized piece of equipment that bonds individual nucleotides together into a strand of nucleic acid. When detecting the presence of viral nucleic acid, the sequences of the synthesized oligo probes spotted on the chip are complementary to known sequences within the viral genome of interest.

Since viral nucleic acids may be in low abundance in a sample, microarray experiments begin with PCR to amplify the sample DNA (Fig. 7.16A). Primers are used that amplify randomly, rather than specifically, so that all DNA in the sample is amplified. Fluorescent nucleotides are incorporated into the amplified DNA. Following amplification, the fluorescent DNA is added to the microarray, and hybridization is allowed to occur between the sample DNA and the oligos on the microarray. Any hybridized DNA will remain bound to the oligos on the chip, while unhybridized DNA is rinsed away. The microarray is read by a machine that measures fluorescence. If fluorescence is present at a particular oligo spot, then the DNA from the sample is bound there and the machine will report a positive result (Fig. 7.16C). The identity of each oligo is known, and so the sequences present in the initial DNA sample can be determined.

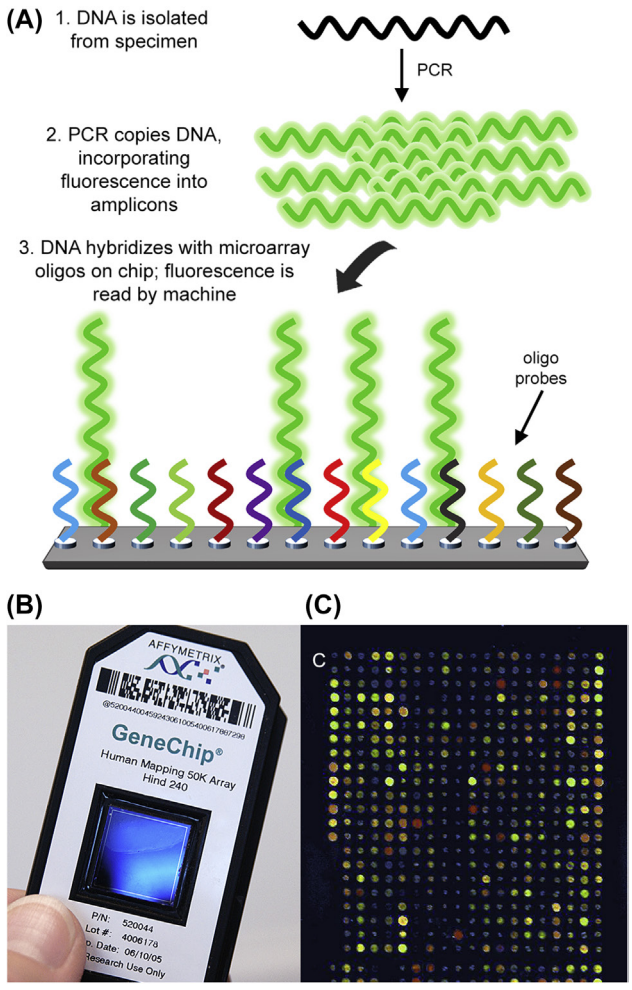

FIGURE 7.16 DNA microarrays. (A) Microarrays work on the principle of hybridization. For microarrays like the ViroChip that identify viruses within a sample, the starting DNA is copied using PCR. Fluorescent nucleotides are incorporated in the process. Then, the fluorescent amplicons are added to the microarray, which has spotted on it thousands of different oligonucleotide probes that each recognize a different viral sequence. If hybridization occurs with the sample DNA, then the virus's nucleic acid was present in the starting sample. A machine measures the fluorescence at each spot. (B) An example of a microarray, found on the blue chip in the middle of the photo. Note the size of the chip, compared to the size of this researcher's thumb. This chip contains 50,000 probes that hybridize to single nucleotide differences within the human genome. (C) Just a small portion (400 oligo spots) of an actual readout of a chip, showing in which spots fluorescence is present. This result used two different samples of cDNA, one labeled green and one labeled red. *(B) and (C) courtesy of Maggie Bartlett and the National Human Genome Research Institute.*

Microarrays can be used for virus identification, but by modifying the oligos on the chip, microarrays can also identify strains of viruses that are particularly virulent or are genetically resistant to certain therapeutics. As long as the genome sequences are known that correspond to these attributes, then an oligo can be made that corresponds to the genetic trait.

Microarray principles can be employed for identification of RNA transcripts or proteins, as well. For example, instead of using DNA oligos to capture nucleic acids, protein microarrays use antibodies to capture different proteins.

An exciting application of the DNA microarray is the **ViroChip,** developed by the DeRisi and Ganem laboratories at the University of California, San Francisco. The ViroChip uses long, 70-nucleotide oligos ("70-mers") as probes that are complementary to known viral sequences. The ViroChip contains over 60,000 probes that detect over 1000 viruses. The researchers included oligos for conserved and novel sequences found in related viruses. In this way, the ViroChip can identify similar viruses based upon conserved genomic regions and can also identify individual subtypes or strains based upon novel nucleotide sequences not possessed by related viruses. The ViroChip can also be used to characterize novel viruses. For example, it was used in 2003 to assist the CDC in identifying a novel virus that was causing deaths in Southeast Asia. The microarray suggested that the unknown virus was a previously unrecognized coronavirus, later to be known as the severe acute respiratory syndrome–associated coronavirus (SARS-CoV).

To create the oligo probes used on the ViroChip, researchers used the genome sequences of any viruses that had been sequenced. **High-throughput sequencing** methods allow for the determination of the nucleotide sequence of potentially any biological entity, including viruses. In the earlier days of viral diagnosis, CPEs were used to classify differences in subtypes or genotypes of viruses. This was replaced by serology and the use of antibodies to determine the viral subtype, and now sequencing of viral genomes provides a definitive differentiation at the nucleic acid level. Currently, over 4600 viral genomes have been completely sequenced, and public databases exist for the genomes of HIV, influenza viruses, dengue virus, and hepatitis C virus, among others. Sequencing also allows us to track the genetic differences between related subtypes and strains of viruses, and it assists scientists in identifying novel viruses by comparing genome sequences with those of known viruses. **Bioinformatics** is the field of study that uses computers to analyze and compare biological data, including genome and protein sequences. Bioinformatics is used to compare viral genome sequences, monitor viral evolution over time, and track virus mutations that appear during epidemics.

There are advantages and disadvantages to every method of diagnosis (Table 7.4), which is why there are a range of tests available for the diagnosis and confirmation of commonly encountered viral infections (Table 7.5).

TABLE 7.4 Advantages and Disadvantages of Various Viral Diagnostic Techniques

Technique	Advantages	Disadvantages
Cell culture	Can be used to detect viruses of unknown identity, cheaper than molecular assays, can distinguish infectious versus noninfectious virus, can be used to amplify a small amount of virus, can be a starting point for other tests	Slow (takes time for virus to replicate), requires specialized technicians, relatively expensive, living cells lead to variability, requirement for specialized equipment (BSCs) and laboratories (BSLs), not all viruses will replicate in cell culture, requires infectious virus
Cytology/histology	Quick because cells/tissues are sent as the specimen, staining assays are fast	May not provide definitive identification of virus, cannot differentiate between strains or subtypes based upon visual examination, requires medically trained personnel, often pathologists
Immunofluorescence assays	Relatively fast to perform, does not require specialized technicians, can definitively identify virus	Requires specialized equipment (fluorescence microscope), antibodies must exist for the virus being detected
Enzyme immunoassay/Enzyme-linked immunosorbent assay	Can be performed within a few hours, easy to perform, can assay for viral antigen or antiviral antibodies, can distinguish between primary and recurrent infection, can be used to diagnose congenital infections	Not available for all viruses, requires specialized equipment (spectrophotometer), low levels may not be detected by the assay
Western blot	Can confirm other tests, relatively quick to perform	Requires specialized reagents and equipment, low sensitivity
Agglutination/hemagglutination reactions	Quick to perform, no specialized equipment necessary, visible read-out	Can be difficult to interpret results, requires specialized reagents, only certain viruses hemagglutinate red blood cells
Lateral flow immunoassay	Can be performed at home or in a clinic, does not require specialized equipment or training, fast, relatively inexpensive	Can result in an indeterminate result, must be verified by other tests, may not distinguish between strains
Polymerase chain reaction	Very little starting material is required, fast, specific, can be performed on nonliving tissues	Requires specialized equipment (thermocycler), knowledge of sequence required for primers, expensive reagents, results are semiquantitative
Real-time PCR	Fast, extremely sensitive, specific, quantitative, can be performed on nonliving tissue, provides immediate results	Requires expensive reagents and specialized equipment, knowledge of sequence required for primers and probe
DNA microarrays	Fast, can test for thousands of different viruses at one time, can be used on nonliving samples, can identify novel viruses	Expensive, requires specialized equipment, knowledge of sequences are required to create oligo probes, does not indicate infectious versus noninfectious virus
High-throughput sequencing	Relatively fast, can provide entire viral sequence, does not require living tissues, can be used to differentiate between closely related viruses, can track viral mutations	Expensive reagents and equipment, requires specialized technicians to interpret results, amount of data generated can be overwhelming and not easily interpreted

TABLE 7.5 Types of Diagnostic Tests Available for Well-Known Viral Infections

Virus	Diagnostic tests available
Adenovirus	Cell culture, IFA
Cytomegalovirus	Cell culture, IFA, qPCR, ELISA (IgM/IgG), DNA sequencing
Dengue virus	ELISA (IgM/IgG)
Enteroviruses	Cell culture, IFA, qPCR
Hepatitis A virus	ELISA (IgM/total)
Hepatitis B virus	ELISA (IgM against surface or core antigen), ELISA (HBV surface and core viral antigens), qPCR
Hepatitis C virus	qPCR, genotyping using RT-qPCR of HCV genome portions, RNA genome amplification and probe hybridization
Herpes simplex virus	Cell culture, IFA, ELISA (IgG)
Human immunodeficiency virus	ELISA (IgM/IgG antibodies, p24 viral antigen), western blot (confirmation), LFIA, RT-PCR then sequencing, genome amplification and probe hybridization
Human papillomavirus	Cytology, genome amplification and probe hybridization
Influenza virus	Cell culture, hemagglutination, IFA
Measles virus	Cell culture, IFA, ELISA (IgM/IgG)
Rotavirus	Electron microscopy, latex particle agglutination
Rubella virus	Cell culture, IFA, ELISA (IgM/IgG)
Varicella zoster virus	Cell culture, IFA
West Nile virus	ELISA (IgM/IgG)

SUMMARY OF KEY CONCEPTS

Section 7.1 Collection and Transport of Clinical Specimens

- Diagnostic tests are paramount in determining the etiology of viral infections.
- Specimens can be collected from a variety of body fluids. The choice of specimen will depend upon the site and stage of infection and whether it is best to test for virus or antiviral antibodies.
- Heparin or EDTA prevents the clotting of blood. Plasma is the liquid portion of nonclotted blood (and so contains clotting factors), while serum is the liquid portion of clotted blood.
- Specimens must be carefully acquired, stored, and transported to ensure integrity of the samples. This is critical to ensure a meaningful and accurate test result.

Section 7.2 Virus Culture and Cell/Tissue Specimens

- Direct diagnostic methods assay for the presence of the virus, while indirect methods test for effects of the virus.
- Cell culture is the process of growing cells or tissues in the laboratory, using an incubator and special culture medium. Cells must be manipulated in at least a class II BSCs to maintain the sterility of the cultures.
- Biological safety cabinets rely upon filtering air through HEPA filters, which filter out 99.97% of particles 0.3 μM in size. Class II BSCs are most often used in research and clinical diagnostic laboratories.
- Certain viruses can cause severe effects and must be handled with caution. BSLs specify which precautions should be taken and are determined by the type of pathogen. They range in stringency from BSL1 to BSL4.
- Cell lines can be infected with patient samples to allow viral replication within the cells. CPEs, such as morphological changes, ballooning, syncytia formation, or inclusion bodies, can help to identify the virus identity. Infected cells can also be used for IFAs.
- Cytology and histology are the staining and microscopic examination of cell and tissue specimens, respectively.

Section 7.3 Detection of Viral Antigens or Antiviral Antibodies

- There are a variety of immunoassays that use antibodies to identify viruses or antiviral antigens. The commercial availability of manufactured antibodies has revolutionized diagnostics.
- Immunofluorescence assays are performed on fixed cells or tissue. Fluorescently labeled antibodies bind to viral antigens present in infected cells. A fluorescence microscope is used to excite the fluorophores so they give off colored light. IHC works in the same way except a colored precipitate is deposited at the site of the antibody.
- ELISAs can be used to verify the presence of viral antigens or antiviral antibodies in liquid patient specimens. In a direct sandwich ELISA, capture antibodies that specifically recognize the viral antigen are coated on the bottom of an ELISA plate with 96 wells. If the patient sample contains the virus, then it will bind to the antibodies and become immobilized to the well. Detection antibodies are conjugated to an enzyme that will cause color change when a substrate is added. An indirect ELISA uses the same principles but coats viral antigen on the plate bottom to detect antiviral antibodies in a patient sample.
- Western blots are sometimes used as a confirmatory test. They are analogous to indirect ELISAs, except the viral antigens are separated by PAGE and transferred to a nitrocellulose membrane before patient samples are added.
- Agglutination reactions use antigen- or antibody-coated latex beads. If the patient sample contains the complementary antibody or antigen, the beads will agglutinate and form a lattice clump. Some viruses naturally hemagglutinate red blood cells.
- LFIAs work like an ELISA in a stick. They rely upon a liquid patient sample traveling through a membrane and encountering antibody-coated beads that accumulate to cause a visible line.
- Plaque assays measure the amount of infectious virus in a sample. It measures the number of plaques formed by allowing a single virion to infect a cell and laterally infect neighboring cells, forming clearings that become visible when the cells are stained. To determine the PFU/mL, the number of plaques is divided by the sample dilution and the volume added to the cells.

Section 7.4 Detection of Viral Nucleic Acids

- Nucleic acid testing is a sensitive and specific way to identify viruses and viral subtypes/strains.
- PCR recapitulates DNA replication in a test tube. Following the isolation of nucleic acid from the clinical specimen, a thermocycler uses heat to separate the two DNA strands. Primers anneal to a target sequence on each strand, and Taq polymerase extends the primer to create the complementary strand. The process is repeated 30–35 times to generate billions of copies of the amplified sequence.
- Real-time PCR uses fluorescence to monitor PCR reactions in real time. It is quantitative because the rate of the reaction can be used to determine the initial starting material.
- DNA microarrays rely upon the hybridization of fluorescently tagged DNA or cDNA to oligo probes coated on a glass slide or silicon chip. These are currently used for research purposes but have great potential for viral diagnosis.
- High-throughput sequencing allows for the rapid determination of nucleotide sequences, including viral

genotypes. It generates nucleic acid sequences that can be analyzed using bioinformatics.

FLASH CARD VOCABULARY

Serology	Histology
Tissue culture	Inclusion body
Cell culture	Immunofluorescence assay
Leukocytes	Fluorescein isothiocyanate
Plasma/serum	Direct versus indirect fluorescent antibody
Direct versus indirect diagnostic methods	Staining
Biological safety cabinet	Immunohistochemistry
HEPA filter	Enzyme immunoassays/ enzyme-linked immunosorbent assays
Laminar flow	
Biosafety level	Direct (sandwich) ELISA
Cytopathic effects	Indirect ELISA
Syncytium	Antiviral antibodies
Cytology	Western blot
Agglutination/hemagglutination reactions	Multiplex PCR
Lateral flow immunoassay	Real-time PCR
Plaque assay	Reporter dye and quencher
Plaque-forming units	Amplification plot
Confluency	DNA microarrays
Nucleic acid testing	Hybridization
Polymerase chain reaction	Oligonucleotides (oligos)
Thermocycler	ViroChip
Primer	High-throughput sequencing
Reverse transcriptase PCR	Bioinformatics
Amplicon	

CHAPTER REVIEW QUESTIONS

1. You are a doctor. A patient shows up in your office that appears to have shingles, which is a reactivation of varicella zoster virus, the virus that causes chickenpox. How would you suggest going about definitively diagnosing her infection?
2. In the 2009 H1N1 influenza pandemic, specimens from patients with potential influenza infections were tested to verify the influenza subtype. What types of specimens might have been collected for such purposes?
3. Describe the observable CPEs that viruses induce in cells.
4. Explain how a class II BSC works to maintain a sterile working environment.
5. You are working with samples that contain human respiratory syncytial virus, which causes coldlike symptoms in adults. Which BSL is most likely required for work with this virus?
6. You have a serum sample, and you would like to verify whether it contains antibodies against a certain virus. Which of the following assays could be used for this? Explain why each is or is not appropriate to use: Immunofluorescence, ELISA, LFIA, cell culture.
7. List the steps involved in performing an indirect ELISA and direct sandwich ELISA. What is each used to measure?
8. You perform a plaque assay with different dilutions of virus, plating 0.1 mL per well of your cells. Your 1/10,000 dilution has no plaques, your 1/1000 dilution has 59 plaques, and your 1/100 dilution has too many plaques to count—they are not distinctive. How many pfu/mL are in your undiluted sample?
9. Describe what happens at each stage of PCR. How is real-time PCR performed differently?
10. You perform qPCR on two patient samples. Both show amplification of viral DNA. One sample crosses the threshold limit at 25 cycles, and the other patient's sample crosses at 32 cycles. Which patient sample had more viral DNA in it?
11. Which type of test would be most effective in determining the entire nucleic acid sequence of a new strain of influenza virus?

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