Molecular Diagnostics

9

Abstract

Effective and early management of diseases requires record of the history, behavioral parameters, and travel information. These are helpful for the diagnosis, prevention, and control of the disease. There have been several advancements in the methods for diagnosing infectious diseases. The wide spectrum of tests such as biochemical evaluation, microbiological tools, immunological and molecular biology techniques, etc., is available. Each type of diagnostic technique is strong and reliable in its own sense but poses certain limitations. These limitations may be complemented by using a combination of tests. Older techniques such as microscopy and culturing of organisms from clinical specimens are error-free but are very labor intensive and extremely time consuming. There is a need to develop rapid and sensitive tests that can be used in both high- and low-resource settings. Molecular diagnostics such as Western blot, ELISA, PCR, DNA, and protein microarrays are revolutionizing the clinical practice of infectious diseases. Their effects are significant in acute-care settings where timely and accurate diagnostic tools are critical for patient treatment decisions and outcomes.

9.1 Disease Pathology and Clinical Spectrum

Effective and early management of diseases requires record of the history, behavioral parameters, and travel information. These are helpful for the diagnosis, prevention, and control of the disease. There have been several advancements in the methods for diagnosing infectious diseases. The wide spectrum of tests such as biochemical evaluation, microbiological tools, immunological and molecular biology techniques, etc., is available. Each type of diagnostic technique is strong and reliable in its own sense but poses certain limitations. These limitations may be complemented by using a combination of tests. Older techniques such as microscopy and culturing of organisms from clinical specimens are error-free but are very labor intensive and extremely time consuming. There is a need to develop rapid and sensitive tests that can be used in both high- and low-resource settings. Molecular diagnostics such as Western blot, ELISA, PCR, DNA, and protein microarrays are

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revolutionizing the clinical practice of infectious diseases. Their effects are significant in acute-care settings where timely and accurate diagnostic tools are critical for patient treatment decisions and outcomes.

9.2 Diagnosis of Bacterial, Viral, and Parasitic Diseases

The diagnosis of these agents is done by using many tests either alone or in combination.

9.2.1 Serological Tests

These are serology-based diagnostic tools. They are more sensitive and specific than microscopic tests. There are two categories of these diagnostic tools that are based on antigen-detection assays and antibody-detection assays. These assays include the Western blotting, enzyme-linked immunosorbent assay (ELISA), and all its derived tests such as the Falcon assay screening test-ELISA (FAST-ELISA), dot-ELISA, hemagglutination (HA) test, indirect or direct immunofluorescent antibody (IFA or DFA) tests, complement fixation (CF) test, and immunoblotting and rapid diagnostic tests (RDTs).

9.2.1.1 Western Blot

In a Western blot, the proteins present in a sample are separated according to their molecular weight by gel electrophoresis. A nitrocellulose membrane is placed on the gel, and with the help of electrical current, the proteins are transferred from the gel to the membrane where they adhere. The pattern of protein separation is maintained in the membrane after transfer. The membrane is then probed with specific antibodies (primary antibodies) to determine the presence of the protein. Often a secondary antibody conjugated to biotin or a reporter enzyme is used to enhance the signal and detect the binding of the primary antibody. This procedure is used mainly to determine the presence of an antigen in biological sample with simultaneous determination of the molecular weight of a protein and measure relative amount of a protein present in different samples (Fig. 9.1).

9.2.1.2 Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA is a diagnostic tool that is used in medicine and other industries to detect and quantify specific antigens. The sample with an unknown amount of antigen is immobilized on a solid support, usually a microtiter plate. This is done either nonspecifically by adsorption or specifically by capture by another antibody specific to the same antigen, in a "sandwich" ELISA. After the antigen is immobilized, the detection antibody is added, forming a complex with the antigen. The detection antibody can be covalently linked to an enzyme or can itself be detected by a secondary antibody that is linked to an enzyme. The plate is developed by adding an enzymatic substrate to produce a visible signal which indicates the quantity of the antigen in the sample (Fig. 9.2).

Both Western blot and ELISA are used to detect HIV infection in the blood. They are called indirect tests as they measure the immune system's response to an infectious agent rather than looking for the components of the agent itself. Since ELISA detects HIV antibodies which the body starts to produce between 2 and 12 weeks after becoming infected with HIV, one should wait for at least 3 months to confirm for HIV AIDS. Western blot is the most common method of testing to confirm positive results from ELISA test. It is used more as a confirmatory test as it is difficult to perform and requires high skills. One advantage of Western blot is that it is less likely to give false-positive results as it can effectively distinguish between HIV antibodies and other antibodies.

9.2.1.3 Falcon Assay Screening Test-ELISA (FAST-ELISA)

This test uses synthetic and recombinant peptides to evaluate antibody responses to an antigen. However, this technique is subjected to the same drawbacks as most serology-based tests. Antibodies raised against a peptide from one protein may cross-react with proteins from other species. Moreover, antibodies raised against a

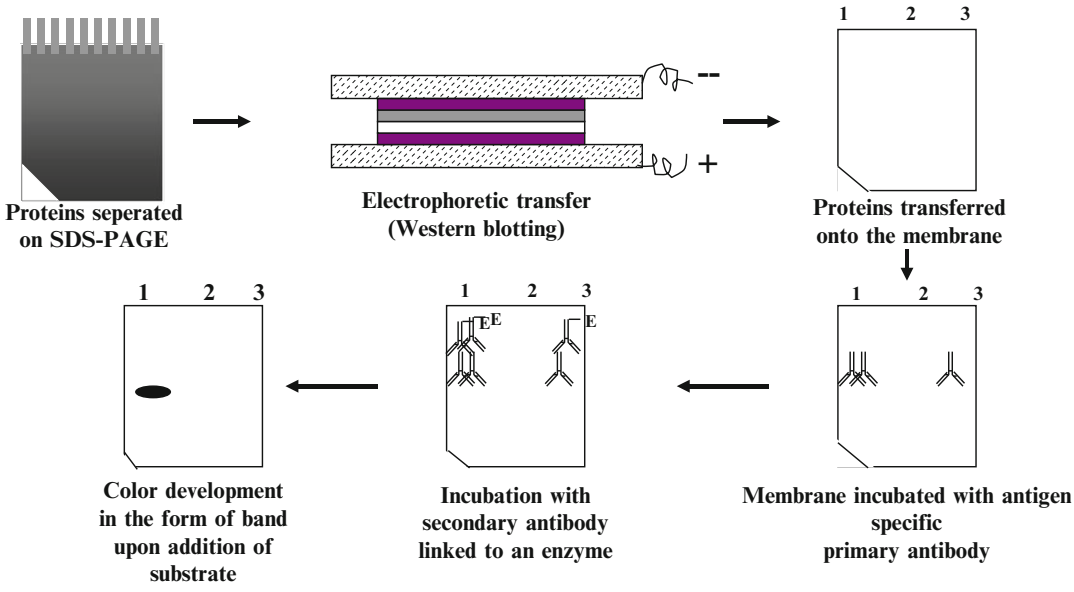

Fig. 9.1 The technique of Western blotting in which proteins separated on SDS-PAGE are transferred from the gel onto a membrane and detected using specific antibodies. Electrophoretic transfer is performed followed by incubation with antigen-specific primary antibody. The blot is washed and subsequently incubated with enzyme-linked secondary antibody. Addition of substrate results in formation of bands, whose intensity is related to the quantity of initial antigen present in the sample. For example, lane 1 and 3 have high and low antigen concentration, where as lane 2 has no detectable antigen

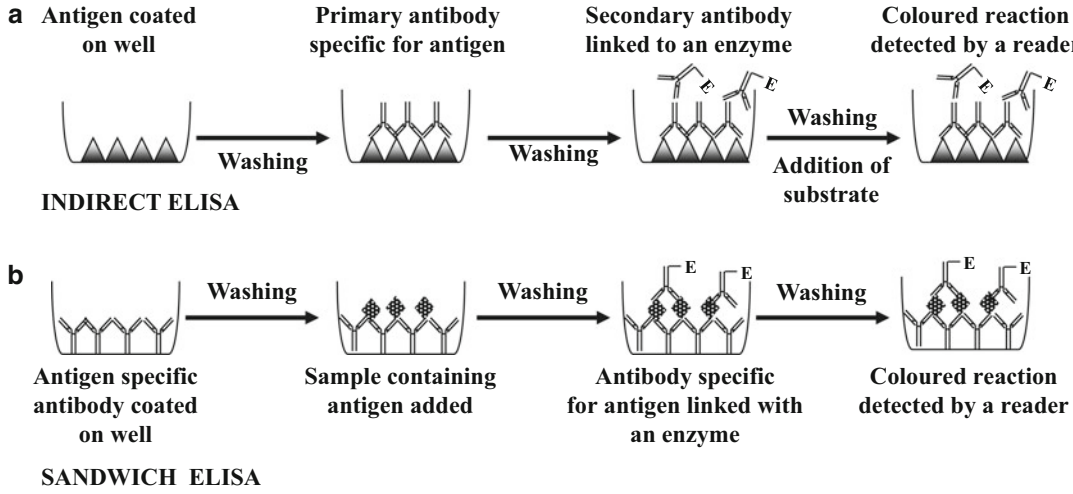

Fig. 9.2 The technique of enzyme-linked immunosorbent assay (ELISA) for the detection of antigen or antibody. (**a**) Indirect ELISA. Here the wells are coated with antigen and incubated with primary or antigen-specific antibody present in the sample. After washing, a secondary antibody linked with an enzyme is added. The binding of the antibody and addition of substrate gives colored product. (**b**) Sandwich ELISA. For authentication of a pathogen, two epitopes are tested by this technique. Antibody specific for a particular epitope of antigen are coated on well followed by the addition of the sample containing antigen. This results in antigen–antibody binding. Binding of another antigen-specific antibody linked with enzyme results in color formation upon addition of the substrate

peptide may react in some assays but not in others as some regions of a peptide may be more immunogenic than others. In the past, the method has been applied to the study of malaria, fasciolosis, schistosomiasis, and taeniasis. Lately it is not used regularly.

9.2.1.4 Dot-ELISA

The main difference between the regular ELISA and the dot-ELISA lies in the surface used to bind the antigen of choice. In the dot-ELISA, the plastic plate is replaced by a nitrocellulose or other paper membrane onto which a small amount of sample volume is applied. The principle is similar to that of immunoblotting. The dotted membrane is incubated first with an antigen-specific antibody followed by an enzyme-conjugated anti-antibody (secondary antibody). The addition of a precipitable, chromogenic substrate causes the formation of a colored dot on the membrane which can be visually read. It is convenient to use, gives rapid results that are fairly easy to interpret, is fast and cost-effective, and hence can be used in the field (e.g., as a dipstick). For all these reasons, the dot-ELISA is extensively used in the detection of human and animal parasitic diseases, including amebiasis, babesiosis, fascioliasis, cutaneous and visceral leishmaniasis, cysticercosis, echinococcosis, malaria, schistosomiasis, toxocariasis, toxoplasmosis, trichinosis, and trypanosomiasis [4].

9.2.1.5 Rapid Antigen-Detection Tests (RDTs)

This test is based on immunochromatographic antigen detection and has been implemented in many diagnostic laboratories as an adjunct to microscopy for the diagnosis of malaria. RDTs consist of capturing soluble proteins by complexing them with capture antibodies embedded on a nitrocellulose strip. A drop of blood sample is applied to the strip and eluted from the nitrocellulose strip by the addition of a few drops of bufer containing a labeled antibody. The antigen–antibody complex can then be visualized directly from the membrane. RDTs are now rapid, stable at temperatures up to 40 °C, easy to use, and cost-effective, thereby providing many advantages over traditional microscopic methods.

9.2.1.6 Luciferase Immunoprecipitation System (LIPS)

This is a modified ELISA-based assay in which serum containing antigen-specific antibodies can be identified by measuring light production. Basically, an antigen of choice is fused to the enzyme reporter Renilla luciferase (Ruc) and expressed as a Ruc-fusion in mammalian cells to allow for mammalian-specific posttranslational modifications. The crude protein extract is then incubated with the test serum and protein A/G beads. During the incubation, the Ruc-antigen fusion becomes immobilized on the A/G beads, which allows the antigen-specific antibody to be quantitated by washing the beads and adding coelenterazine substrate and measuring light production. Some of the advantages of the LIPS technology include its rapidity and accuracy in detecting infected patients. Sensitivity is improved in part by the use of mammalian cells which produce fusion antigens free of contaminating bacterial proteins. In addition, low backgrounds are produced compared to the ELISA.

9.2.1.7 Antibody-Based Diagnosis: Monoclonal Antibodies as Diagnostic Reagents

Monoclonal antibodies (mAb) are derived from identical immune cells that are clones of unique parent cells and can bind to a specific epitope (for further details, refer to Chap. 14). They have been extensively used in biomedical and microbiological research as tools for diagnosis of diseases such as hepatitis, AIDS, influenza, herpes simplex virus infection, chlamydial infection, and treatment of cancer [7, 9]. The monoclonal antibodies being directed against single epitopes are homogeneous and highly specific and can be produced in unlimited quantities. Monoclonal antibodies have tremendous applications in the field of diagnostics, therapeutics, and targeted drug delivery systems, not only for infectious

diseases caused by bacteria, viruses, and protozoa but also for cancer and metabolic and hormonal disorders.

In 1975, Kohler and Milstein invented the hybridoma technology. The key idea was to use a line of myeloma cells that had lost their ability to secrete antibodies, fuse these cells with healthy antibody-producing B cells, and select for the successfully fused cells. In hybridoma technology, a myeloma cell rendered drug sensitive through mutation in a growth essential gene, hypoxanthine guanine phosphoribosyl transferase (HGPRT), is chemically fused with immune cells from a host immunized with the antigen of interest, and the resulting cells are grown in medium containing the selective drug. Since the immune cells have a short life span in tissue culture and the myeloma cells are drug sensitive, the only cell that will survive are those myeloma cells which obtained a normal HGPRT gene from the immune cells. Such cells also have a high likelihood of carrying the immune cell's antibody gene resulting in the generation of a hybridoma that can grow continuously in vitro and secrete a single monoclonal antibody (Fig. 9.3).

The diagnosis of any infectious disease often requires the demonstration of the causative organism or presence of a specific antibody. Specific antibody-based tests identify the pathogens associated with the disease. MAbs recognizing unique antigenic determinants on pathogens are developed. This restricted reactivity allows for precise identification of the organism of interest which is the major advantage of MAbs over polyclonal antisera. In case of a pathogen occurring as subtype defined by unique antigenic differences, specific MAbs can be used, whereas conventional antisera needs laborious absorption to remove cross-reactive antibodies. Because of the specificity, homogeneity, and unlimited availability of the MAbs, vast amount of work has been carried out on the production/development

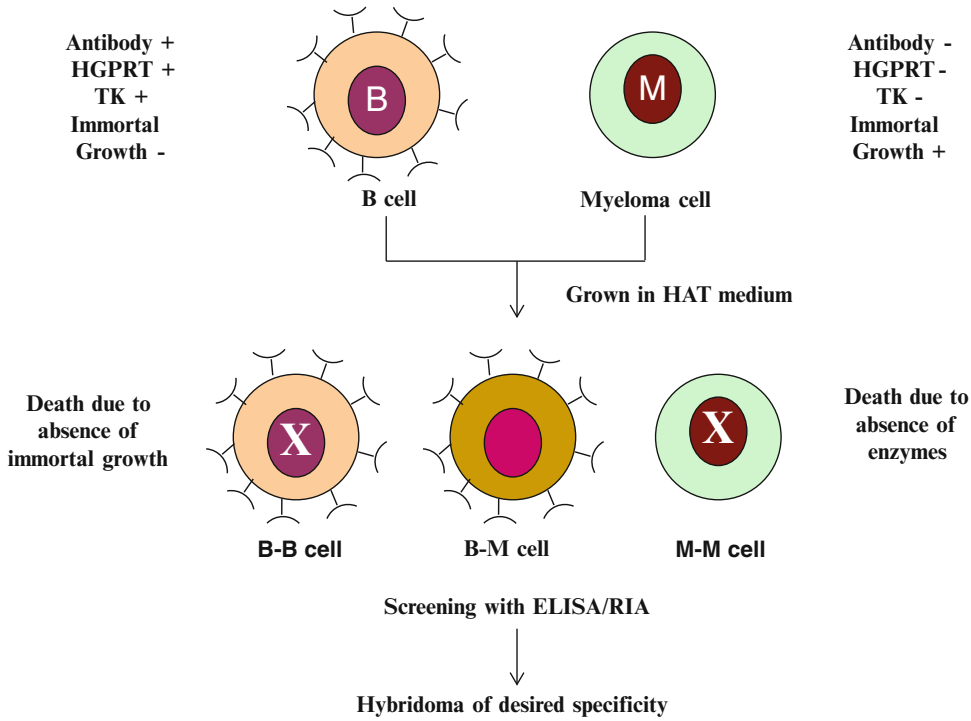

Fig. 9.3 Hybridoma technology. Antibody-secreting B cells that are positive for enzymes (HGPRT and TK) are fused with myeloma cells that have immortal growth. After fusion, the cells are selected on HAT medium. Only B-M fusion cells would survive in presence of aminopterin (HAT) present in the HAT medium. These cells are further screened for desirable specificity of the antibody produced by ELISA or RIA and are used for large-scale production of antibody

of MAbs diagnostic reagent tests against various pathogenic agents.

The immuno-diagnoses of protozoan and parasitic diseases have significantly been improved by MAb technology because the tests involving MAb as diagnostic reagents overcome the limitations of polyclonal antibodies. MAbs were found to be extremely useful in the rapid outbreak of East Coast fever (ECF). MAbs of diagnostic value have also been developed against *Trichomonas vaginalis*, *Leishmania donovani*, *Trypanosoma congolense*, and *Babesia bovis*. Development of monoclonal antibodies for the detection of *Mycoplasma pneumonia* and plum pox virus has been reported.

9.3 Nucleic Acid-Mediated Tests

9.3.1 PCR and Array-Based Techniques in Diagnosis

PCR is the most well-developed molecular technique that has not only been successfully applied for several wide-ranged clinical diagnoses but also has great potential for clinical applications, including specific or broad-spectrum pathogen detection, evaluation of emerging novel infections, surveillance, early detection of biothreat agents, and antimicrobial resistance profiling. PCR-based methods may also be cost-effective relative to traditional testing procedures. Further advancement of technology is needed to improve automation, optimize detection sensitivity and specificity, and expand the capacity to detect multiple targets simultaneously (multiplexing). PCR is the most sensitive and rapid method of detecting pathogens in clinical samples. It is very useful as some of the microorganisms are not easily culturable in vitro or has a very long incubation time. Under these conditions, the diagnostic value of PCR is very important [12].

Traditional PCR procedure includes amplification of specific genes (Fig. 9.4) of the microorganisms and running the product on a gel. The presence of a microbe is confirmed by the presence of a band of appropriate size. Nested, multiplexed, and real-time PCR (RT-PCR) are used for efficiency and quantitation.

Multiplexed PCR allows the detection of multiple sequences in the same reaction tube proving useful in the diagnosis of several infections simultaneously (Fig. 9.5).

RT-PCR system, unlike conventional PCR, allows for the quantification of the original template's concentration through the use of various fluorescent dyes and primers. The concentration is measured through comparison to standard curves. This eliminates the need to visualize the amplicons by gel electrophoresis, thereby greatly reducing the time, risk of contamination, and the introduction of false-positives.

PCR is used to diagnose the presence of several opportunistic pathogens in the cerebrospinal fluid of HIV patients or multiple sclerosis patients [2, 11]. The viral infections that can be determined by this method are *Herpes simplex virus* (type 1 and 2), *Varicella zoster virus*, *Cytomegalovirus*, *Epstein–Barr virus*, and *Japanese encephalitis* virus. Bacterial infection such as *Chlamydia pneumoniae* is also identified. *Mycoplasma* sp. is very difficult to cultivate in laboratory; hence, PCR method is the only reliable method to identify the presence of the samples [8]. DNA probes consisting of cloned ribosomal RNA genes, cDNA to mycoplasmal rRNA, synthetic 16S rRNA oligonucleotide sequences, or cloned mycoplasmal protein genes have been developed and applied as diagnostic tools in a variety of human and animal mycoplasma infections.

9.3.2 Loop-Mediated Isothermal Amplification (LAMP)

Is a unique amplification method with extremely high specificity and sensitivity able to discriminate between a single nucleotide differences. It is characterized by the use of four different primers specifically designed to recognize six distinct regions on a target gene, with amplification only occurring if all primers bind and form a product (Fig. 9.6). The reaction occurs at a constant temperature using strand displacement activity of

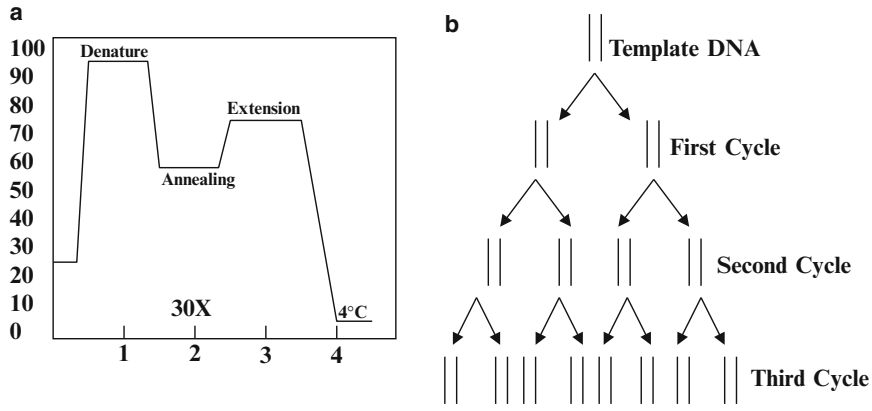

Fig. 9.4 Polymerase chain reaction (PCR). (**a**) shows the PCR cycle where the DNA sequence is amplified using appropriate primers and temperature conditions (denaturation, annealing, and extension). After each cycle, the DNA amplification is shown as in (**b**)

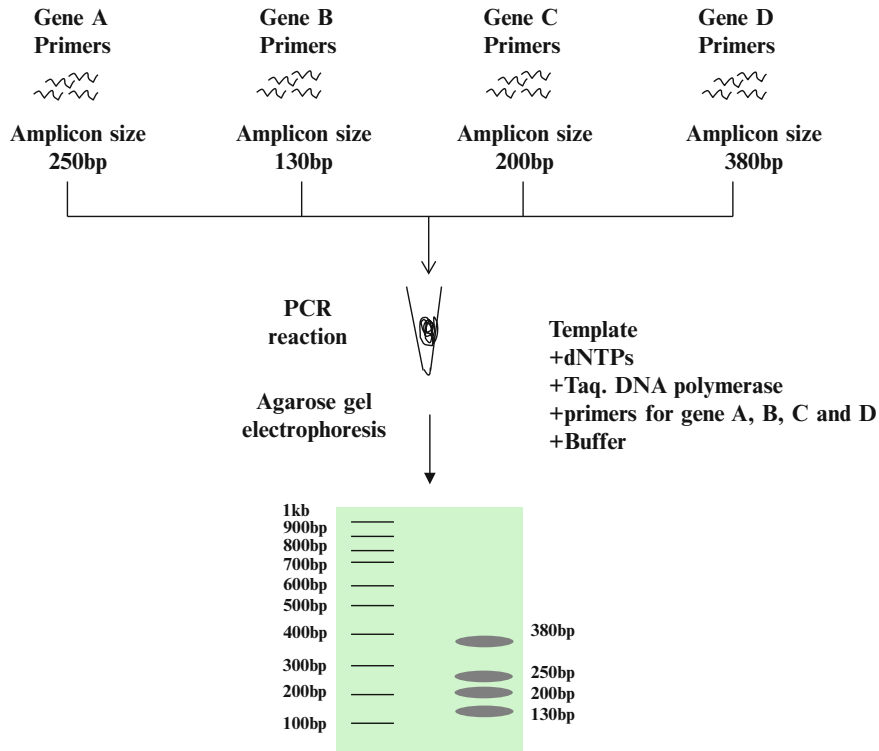

Fig. 9.5 The figure explains multiplex PCR reaction technique. Multiple PCR reactions can be performed in the same tube when the product size of different target amplicons are substantially different from each other and the reaction conditions for all the PCR are similar. The primers for gene A to D, specific for different pathogenic agents, are put together in the same tube and the PCR products are analyzed by agarose gel electrophoresis showing different sized bands

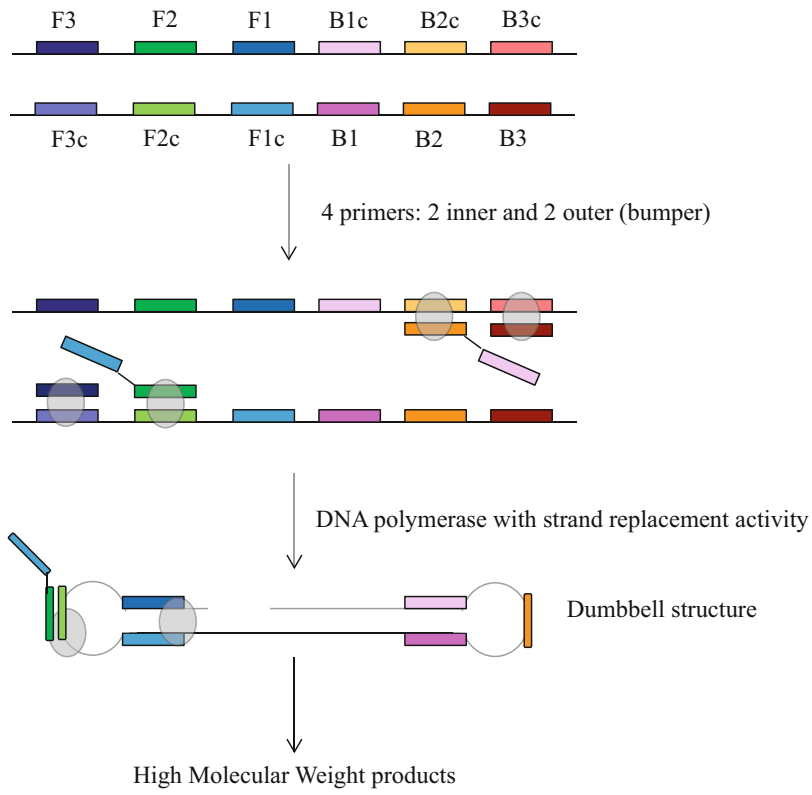

Fig. 9.6 LAMP PCR. In this technique, DNA polymerase with strand displacement property is used to produce single-stranded loop-like templates. 4 primers are used: 2 inner primers and 2 outer primers or bumper primers. The dumbbell-shaped intermediates formed increase the amplification efficiency and the entire reaction takes place at 65° centigrade

DNA polymerase [10]. Amplification and detection takes place in a single step at a constant temperature (65°). It does not require expensive thermo cyclers. The corresponding release of pyrophosphate causes turbidity that is detected visually. Sometimes DNA-intercalating dye is also used. This has been applied for rapid detection of several DNA and RNA viruses such as West Nile and SARS virus. It has also been used for the identification of several parasites.

9.3.3 Luminex xMap Technology

Molecular-based approaches based on nucleic acids offer greater sensitivity and specificity over the existing diagnostic tests. They permit the detection of infections from very low titer samples including those from asymptomatic patients. Luminex technology is a bead-based flow-cytometric assay that allows the detection of various targets simultaneously. The microsphere beads can be covalently bound to antigens, antibodies, or oligonucleotides that will serve as probes in the assay. Up to 100 microspheres are available, each emitting unique fluorescent signals when excited by laser, therefore allowing the identification of different targets. This method has been successfully used for detecting *Cryptosporidium* species. *C. hominis* and *C. parvum* has a single nucleotide difference in the microsatellite-2 region (ML-2) that can be identified only by sequencing which is very time consuming and labor intensive. They can be detected and distinguished by this technology.

However, there are several drawbacks of these methods regarding clinical samples, as PCR is susceptible to inhibitors, contamination, and experimental conditions. The sensitivity and specificity of a PCR assay is dependent on target genes, primer sequences, PCR techniques, DNA extraction procedures, and PCR product detection methods. These might not be optimal in clinical specimens such as blood, urine, sputum, cerebrospinal fluid (CSF), and others. The PCR conditions need to be carefully evaluated and the

results confirmed microbiologically. PCR is used for the diagnosis of *HIV-1*, *Hepatitis B and C viruses*, *Human papillomavirus*, *Chlamydia trchomatis*, *Neisseria gonorrhoeae*, *Cytomegalovirus*, *Mycobacterium tuberculosis*, and many others.

9.3.4 Single Nucleotide Polymorphism and Disease Association

Single nucleotide polymorphisms or SNPs (pronounced as snips) are tiny variations in an individual's genetic code. SNPs occur when a single nucleotide (A, T, G, or C) is substituted for another between the members of the same species or between two chromosomes of the same person. When the DNA sequence of a gene differs by only one nucleotide between two individuals, they are called as alleles.

SNP analysis can be done in a single step by using genomic DNA and PCR method (Fig. 9.7). A single SNP analysis can be done by using a specific primer attached to a fluorescence marker, also known as a quenching probe or Q-Probe. When the primer binds with a specific DNA sequence, the fluorescence is quenched due to association with guanine residue. When it dissociates, the fluorescence is acquired. When the primer binds to the wild-type allele, the dissociation occurs at a higher temperature, whereas in a mutant allele, the binding is weak and dissociation takes place at a lower temperature. This change in dissociation curve is analyzed. Two different colors can be used for multiplex analysis.

SNPs occur due to mutation, recombination, and natural selection. SNPs may occur in coding region of genes, in noncoding regions of genes, or in intergenic regions. They are classified into different categories.

9.3.4.1 Synonymous Polymorphism or Silent Mutation

Due to degeneracy of genetic code, the amino acid sequence of the polypeptide might not change. These are known as synonymous polymorphism.

9.3.4.2 Non-synonymous or Replacement Polymorphism

When the changes produce different polypeptides, they are known as non-synonymous or replacement polymorphism. This may result in missense mutation, where a different amino acid is produced, or nonsense mutation, where there is a premature stop codon. Lot of disease mutations are caused by replacement polymorphism.

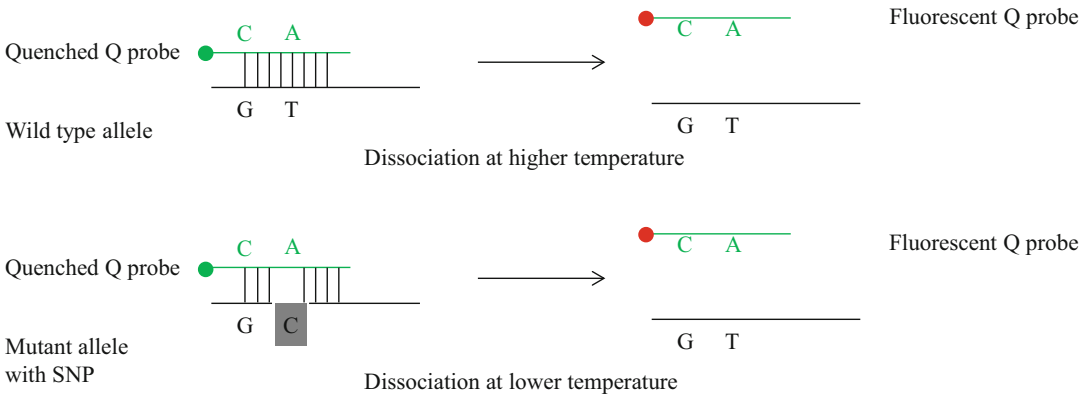

Fig. 9.7 Single nucleotide polymorphism (SNP) analysis. Synthetic oligonucleotide attached to fluorescence marker is incubated with genomic DNA. The fluorescence is quenched due to association to guanine residue. When it dissociates at high temperature, the fluorescence is restored. The dissociation temperature is related to the complementarity of the sequence. The wild type is 100% complementary and hence has a higher dissociation temperature where as the mutant allele with SNP has a low dissociation temperature5 (Source: http://www.aist.go.jp/aist_e/latest_research/2005/20050405/20050405.html)

9.3.4.3 e-SNPs or Expression SNPs

SNPs occurring in noncoding region might affect gene splicing, transcription factor binding, m-RNA degradation, or mutate noncoding RNA.

9.3.4.4 Importance of SNPs

About 99.9 % of DNA sequences are identical between individuals of same species. Out of 0.1 % variation, around 80 % is due to SNPs. Thus they bring about diversity among individuals. This trait is used for DNA fingerprinting in forensic science.

Several diseases are caused by genetic variations in an individual. Genetic factors are responsible for susceptibility and disease progression. SNP profile or haplotype associated with a disease trait may reveal relevant genes associated with a disease state. It provides understanding of many polygenic diseases. In future there are chances that by viewing the SNPs profile of an individual, the physicians might be able to find out the risks associated and plan a personalized medicine. SNPs help in determining the likelihood of a person to develop a particular disease. One of the genes associated with Alzheimer's disease in apolipoprotein E or *ApoE*. It contains two SNPs that result in three possible alleles for this gene: E2, E3, and E4. Each allele differs by one DNA base, and the protein product of each gene differs by one amino acid. Each individual inherits one maternal copy of *ApoE* and one paternal copy of *ApoE*. A person who inherits at least one E4 allele has a greater chance of developing Alzheimer's disease, whereas inheriting the E2 allele reduces the likelihood of developing Alzheimer's. SNPs are not absolute indicators of disease development. *ApoE* is just one gene that has been linked to Alzheimer's. Like most common chronic disorders such as heart disease, diabetes, or cancer, Alzheimer's is a disease that can be caused by variations in several genes. The polygenic nature of these disorders is what makes genetic testing for them so complicated.

9.4 Protein Microarray

Protein microarrays are tools that can be used in both translational as well as basic research. Protein chips can be used for a variety of applications including identification of protein–protein interactions, protein–phospholipid interactions, and substrates for protein kinase. They are used for clinical diagnosis and disease state progression. They can be used to phenotype leukemia cells, identify new protein–protein interactions, screen entire proteomes, and profile hundreds of patient samples. Several arrays are available for specific use. They have been graphically represented in Fig. 9.8. Some of them are discussed here:

9.4.1 Proteomic Arrays

These are high-density arrays and are used to identify novel proteins and protein–protein interactions (Fig. 9.8a). The array library is usually a high-density expression library and the probes are either directly labeled with fluorophores or are tagged with labeled antibodies.

9.4.2 Microspot ELISA and Antibody Arrays

These are used for quantitative profiling of protein expression in clinical samples and cell culture (Fig. 9.8b). These are low-density arrays. Known antibodies are arrayed to capture antibodies from unknown samples. The antigens are either labeled directly or are attached to a secondary antibody. The latter gives a sandwich assay similar to ELISA.

9.4.3 Single-Capture Antibody Arrays

It consists of multiple known antibodies arrayed on a solid surface (Fig. 9.8c). It is used to profile the presence of known antigens from a pool of

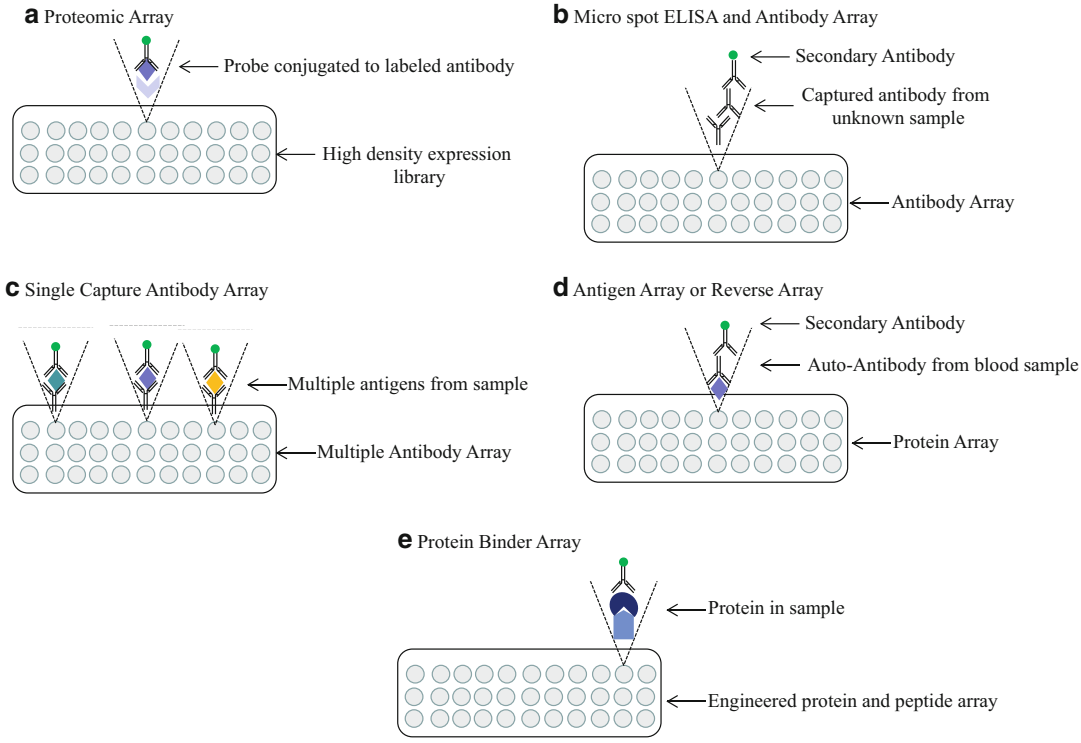

Fig. 9.8 Protein microarray. (**a**) Proteomic array. High-density expression library, probed with samples. This identifies protein–protein interactions. (**b**) Microspot ELISA and antibody arrays. Known antibodies are arrayed to capture antibodies from unknown samples. (**c**) Single-capture antibody arrays. Multiple known antibodies are arrayed on a solid surface, used to profile the presence of known antigens from a pool of samples. (**d**) Antigen arrays or reverse arrays. These are used to detect autoantibodies in samples. These are low-density arrays and are probed with serum or plasma. (**e**) Protein binder assay. Engineered proteins and peptides with various binding motifs are arrayed and are probed with complex samples. Detection with known antibodies helps to identify new binding sites and interactions

samples. Normal and disease samples are used. They are either labeled directly or with haptens.

9.4.4 Antigen Arrays or Reverse Arrays

These are used to detect autoantibodies in clinical and research samples. These are low-density arrays and are probed with serum or plasma (Fig. 9.8d). Reverse arrays are used to probe hundreds of samples to detect the presence of few antibodies. Cell lysates, plasma, and serum are arrayed and are probed with few known antibodies.

9.4.5 Microarray Western

This is an alternative strategy where samples containing several proteins are arrayed on slide and probed with labeled antibodies. Level of number of proteins can be measured simultaneously.

9.4.6 Protein Binder Arrays

This is used to identify novel protein-binding motifs and protein–protein interactions (Fig. 9.8e). Engineered proteins and peptides

with various binding motifs are arrayed and are probed with complex samples. Detection with known antibodies helps to identify new binding sites and interactions.

9.5 Isolation, Processing, and Profiling of Proteins and Other Molecules Associated with Disease

Proteomic studies can provide substantial information about clinical state of a disease as they are the final molecular machines of biological processes. They can be used as biomarkers for disease states. Diagnostics use protein and peptide biomarkers from body fluids. All proteomic-based diagnostic efforts seek to identify biomarkers that, alone or in combination, can distinguish between "case" and "control" groups. This can be done in several ways.

9.6 Profiling and Identification of the Protein

9.6.1 Two-Dimensional Gel Electrophoresis

This is a method to identify proteins and peptides in their natural form. Here the proteins are resolved in the first dimension based on pH (a process called isoelectric focusing) and in the second dimension by their molecular weight. This technique is labor intensive.

9.6.2 Mass Spectrometry (MS)

This is an analytical technique where mass-to-charge ratios of particles are measured. It is used to determine the composition of peptides. Proteins from body fluids can be proteolytically cut into small pieces. They are ionized usually to cations by removal of electron. These charged particles are then separated according to their charge and mass. The separated ions are measured and displayed. The resulting spectra can be compared with other peptides in the data base (Fig. 9.9). But in this approach it is difficult to quantitate and study the protein modifications.

9.6.2.1 Matrix-Assisted Laser Desorption Ionization Time-Of-Flight Mass Spectrometry (MALDI-TOF MS)

It is a relatively novel technique in which a coprecipitate of a UV light-absorbing matrix and a biomolecule is irradiated by a nanosecond laser pulse. Most of the laser energy is absorbed by the matrix, which prevents unwanted fragmentation of the biomolecule. The ionized biomolecules are accelerated in an electric field and enter the flight tube. During the flight in this tube, different molecules are separated according to their mass-to-charge ratio and reach the detector at different

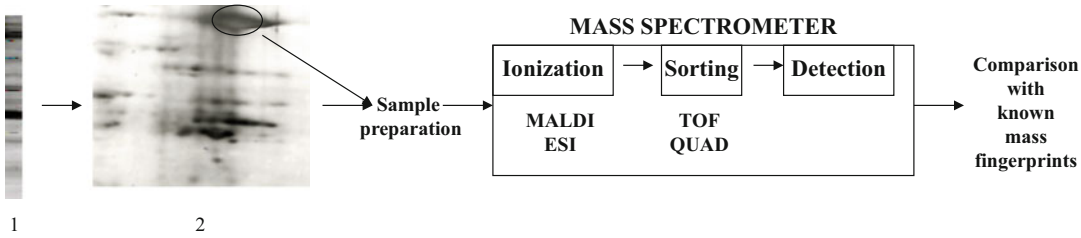

Fig. 9.9 The usage of proteomics approach for diagnostics or profiling. (*1*) First dimensional isoelectric focusing (IEF) gel is used to separate the sample components according to their isoelectric point. (*2*) Second dimensional SDS-PAGE is used which further separates the proteins according to their molecular mass. Sample spots obtained are isolated and prepared for application in mass spectrometer (MS). MS consists of ionization device as MALDI or ESI and mass sorting device as TOF or QUAD and detection is done by a detector. After peptide mass fingerprint is obtained, it is analyzed through comparing the experimentally determined peptide mass fingerprint with known and virtual mass fingerprints using bioinformatics tools

times. In this way each molecule yields a distinct signal. The method is used for detection and characterization of biomolecules, such as proteins, peptides, oligosaccharides, and oligonucleotides, with molecular masses between 400 and 350,000 Da. MALDI-TOF is used for identifying bacterial strains in clinical microbiology laboratories.

The development of automated, high-throughput proteomic technologies such as MS and MALDI-TOF has enabled large numbers of clinical samples to be analyzed simultaneously in a short time. These platforms have made "population-based proteomics" feasible for the first time.

9.7 Nucleic Acid Amplification Technologies (NAAT)

With the use of NAAT, it is now possible to have many copies of target DNA and the technique has advantage of being sensitive, specific, and rapid. It targets the conserve region of the target species. The NAAT test may be planned which would be able to detect single species, strain, or resistance-inducing mutation. Using broad-spectrum probes, the broad categories of the organism may be detected. NAAT has been successfully used in the diagnosis of infective endocarditis as compared to culture technique even when culture reports were negative. In patients with negative sputum smears, the tests based upon NAAT were quiet useful in the clinical diagnosis of tuberculosis.

9.8 Ethics in Molecular Diagnosis

Genetic factors contribute to the risk of several diseases. Genetic testing clarifies the risk of a person suffering from a disease or passing it down to the next generation. However no genetic test can give an absolute answer. It is always percent chance to inherit a disorder. Genetic testing is a rapidly emerging field. There are several disorders that have adult onset and worsen over time. Hence is it advisable for individuals with high risk to undergo genetic testing and counseling. Tests are available for several genetic disorders and cancers. Some of them are sickle cell anemia, Down's syndrome, Huntington's disease, cystic fibrosis, breast cancer, and phenylketonuria. There are three forms of genetic testing. They are diagnostic testing, carrier testing, and predictive testing. Diagnostic testing involves identification of a current disease state. This includes prenatal and newborn genetic testing. Carrier testing includes if an individual carries a particular genetic trait that he or she can pass on to the next generation. Predictive testing determines if a person carries a mutation that can have a late onset of a disorder.

However, genetic testing gives rise to several ethical issues [3]. It might cause potential discrimination regarding social acceptability, job or employment availability, and health insurance coverage. Prenatal testing for genetic disorder may lead to abortion of a fetus. Carriers of genetic mutations ethically should disclose the fact to their life partner or their siblings. But he or she might face social isolation. He or she might not be able to marry and start a family. Similarly if a person is at risk of a late onset of a genetic disorder, the employer might not be willing to hire him or her. The health insurance companies would not want to pay for the medical expenses or might increase the premium [5, 6].

One should also keep in mind that genetic testing cannot give all the answers. For example, it cannot tell about the exact time of onset, penetrance, or person to person variation of a disorder. There are several issues regarding the ethical consideration of genetic testing. Until and unless there are clear laws to protect the individuals, privacy and confidentiality of genetic information should always be protected and individuals wish to be tested or not should be respected [1].

9.9 Chapter End Summary

- Biotechnology has played a very important role in diagnosis and treatment of various bacterial, fungal, viral, and parasitic diseases. It

has also helped in identification of early stages of cancer.
- The advancement in molecular techniques has helped in identification of biomarkers that signifies early development and progress of a disease.
- The various tests like serology-based tests and nucleic acid-based tests are diagnostics, but preliminary data from traditional microbiology-based methods are also helpful.
- The serology-based tests may be done either by targeting antigen or antibody and gives an indication about the current state of the disease.
- The tests based upon DNA detection can indicate the presence or absence of the target pathogenic agent but sometimes are unable to predict about the current status of infection.
- The usage and development of monoclonal antibodies have made tremendous advances in biomedicine.
- The usage of nucleic acid-based methods has helped in identifying the presence of certain microorganisms in samples which were earlier believed to be noninfectious.
- The profiling using proteomics tools supplemented with mass spectrometry have helped to characterize many pathogen-specific factors which might be of interest in diagnostics and therapeutics.

Multiple Choice Questions

1. The commonly used method for diagnosis of bacterial infection is:
   (a) Gram staining
   (b) Antibiotic sensitivity
   (c) Biochemical test
   (d) All of the above
2. In indirect ELISA the primary antibody is:
   (a) Enzyme labeled
   (b) Against constant region of antibody
   (c) Fluorescently labeled
   (d) Against antigenic epitope
3. In reverse western technique:
   (a) The sample is affixed on slide.
   (b) The targets are fixed on slide.
   (c) Both the sample and targets are fixed on slide.
   (d) None of them is fixed on slide.
4. PCR is used for detection of:
   (a) Infection with high titer
   (b) Presence of infectious agent
   (c) Antibody
   (d) Antigen
5. Monoclonal antibodies are used in diagnostics because:
   (a) They can be manufactured by hybridoma.
   (b) They can be prepared by plasma cells of the body.
   (c) They are against a specific target.
   (d) None of the above.
6. There is a requirement of engineering of antibody because:
   (a) Engineering makes the antibody more effective.
   (b) Engineering reduces the immune reactions of therapeutic antibody.
   (c) Engineering makes it more specific for its target.
   (d) All of these.
7. The positive PCR reaction specific for a pathogen indicates:
   (a) The pathogen is active.
   (b) The pathogen is not active.
   (c) The presence of the pathogen.
   (d) All of the above.
8. The purpose of a multiplex PCR is:
   (a) Determination of molecular size of the amplicon
   (b) Determination of the activity of the pathogens
   (c) Determination of the presence of a number of pathogenic nucleic acid
   (d) All of the above
9. A specific sequence analyzed by PCR-RFLP in two individuals showed the presence of one band in one and two bands in the other, because of:
   (a) Presence of microsatellite

(b) Presence of satellite
(c) Presence of SNP
(d) Presence of all of them

10. The proteomics studies have become feasible due to:
(a) PCR reaction
(b) RT-PCR
(c) Mass spectrometry
(d) DNA microarray

Answers

1. (a); 2. (d); 3. (b); 4. (b); 5. (c); 6. (b); 7. (c); 8. (c); 9. (c); 10. (c)

Review Questions

Q1. How has the advancement in biotechnological techniques helped in diagnosis of the diseases?
Q2. Discuss a few serological tests for the diagnosis.
Q3. What is the importance of PCR in pathogen detection?
Q4. How are the proteomic assays helpful in aiding diagnostics?
Q5. Discuss DNA microarray technology.
Q6. What is MALDI-TOF?

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Some Selected Resources

http://en.wikipedia.org/wiki/Monoclonal_antibodies
http://en.wikipedia.org/wiki/Single-nucleotide_polymorphism
http://eubios.info/index.html
http://www.eubios.info/india/BII5.HTM
http://www.differencebetween.com/difference-between-elisa-and-vs-western-blot/
http://www.nature.com/scitable/topicpage/diagnostic-testing-and-the-ethics-of-patenting-709
http://www.nature.com/scitable/topicpage/ethics-of-genetic-testing-medical-insurance-and-651
http://www.nature.com/scitable/topicpage/human-testing-the-eugenics-movement-and-irbs-724
http://www.whatman.com/ProteinMicroarrays.aspx