Molecular Pathology – Review

(Author Aastha Acharya)

Molecular pathology is based on the principles, techniques, and tools of molecular biology as they are applied to diagnostic medicine in the clinical laboratory. Molecular biology methods were used to elucidate the genetic and molecular basis of many diseases, and these discoveries ultimately led to the field of molecular pathology. As molecular research identifies the most fundamental causes and markers of disease, clinical testing of human and pathogen genetic material has become routine in laboratory medicine. Eventually the insights these tools provided for laboratory medicine were so valuable to the armamentarium of the pathologist that they were incorporated into pathology practice. Molecular pathology is a natural extension of anatomic and clinical pathology. As molecular research identifies the most fundamental causes and markers of disease, clinical testing of human and pathogen genetic material has become routine in laboratory medicine. Gene products, such as proteins and polypeptides, are molecules and could technically be classified within "molecular pathology."

Brief History

Historically the touchstone of diagnostic pathology has been the histologic appearance of diseased and normal tissues when stained with conventional stains, usually hematoxylin and eosin. Developments in molecular diagnostics primarily reflect technological breakthroughs in molecular biology. Molecular pathology techniques are rooted in fundamental molecular biology discoveries of the 1940s–1980s, The clinical laboratory application of molecular biology techniques would not be possible without the discovery by Griffith and Avery that nucleic acid is the genetic material. The foundation of work by Chargaff and Franklin was capitalized on by Watson and Crick, who elucidated the structure of DNA. Understanding DNA structure is seminal to understanding nucleic acid hybridization, which is central to almost all molecular methods used in the clinical molecular pathology laboratory. Additionally, work by Nirenberg (unraveling the genetic code), Wilcox, Smith, Nathans, and others (use of restriction endonucleases for DNA manipulation), Baltimore and Temin (discovery of RNA-dependent DNA polymerase or reverse transcriptase), Britten and Davis (hybridization kinetics), Kornberg and Okazaki (work on DNA polymerases and DNA replication, respectively), Southern

(development of solid-phase DNA hybridization, i.e., the Southern blot), Sanger, Maxam, and Gilbert (development of DNA sequencing), Mullis (discovery of PCR for in vitro nucleic acid amplification), and their scientific collaborators and competitors led to a refined understanding of how DNA may be manipulated in vitro for research and ultimately clinical molecular testing purposes.

PRACTISE OF MOLECULAR PATHOGENESIS

Molecular diagnostics is associated with virtually all clinical specialties and is a vital adjunct to several areas of clinical and laboratory medicine, but is most predominantly aligned with infectious disease, oncology, and genetics. While the unique disease principle emphasizes the individuality of each disease process, molecular disease classification attempts to identify commonality in disease features, subgroup disease based on these shared characteristics, and predict disease evolution, progression, and therapeutic response. Molecular diagnostics is the outcome of the fruitful interplay among laboratory medicine, genomics knowledge, and technology in the field of molecular genetics, especially with significant discoveries in the field of molecular genomic technologies. All these factors contribute to the identification and fine characterization of the genetic basis of inherited diseases which, in turn, is vital for the accurate provision of diagnosis. High-throughput methods, such as next-generation sequencing or genome-wide association studies, provide invaluable insights into the mechanisms of disease, and genomic biomarkers allow physicians to not only assess disease predisposition but also to design and implement accurate diagnostic methods and to individualize therapeutic treatment modalities.

Some of the disease entities are described below where molecular pathology plays vital role in its identification and how this novel therapeutic helps are:

Infectious Diseases

1. Flavivirus Disease :

Flavivrus such as Hepatitis C Virus was first recognized in 1989 using recombinant technology to create peptides from an infectious serum that were then tested against serum from individuals with non-A, non-B hepatitis. This approach resulted in the isolation of a section of the HCV genome. Most clinical testing for HCV infection begins with detection of antibodies against HCV proteins. Most false negative results of the anti-HCV assay occur in the setting of

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immunosuppression, such as with human immunodeficiency virus (HIV) infection, or in renal failure. Qualitative assays are designed to determine the presence or absence of HCV RNA, without consideration of actual viral load, which are proven to be more efficient.

2. Enteroviral Disease:

Enteroviruses (EV) are a general category of singlestranded, positive-sense, RNA viruses of the Picornaviridae family, which includes the subgroups enteroviruses, polioviruses, coxsackieviruses (A and B), and echoviruses. Until recently, a diagnosis of EV infection was based on clinical presentation and the isolation of virus in cell culture from throat, stool, blood, or cerebrospinal fluid (CSF) specimens. EV detection by culture methods is insensitive and takes too long to be useful in clinical management decisions. By contrast, reverse transcription- PCR (RT-PCR) is more rapid and sensitive and has been used to detect EVRNA in CSF, throat swabs, serum, stool, and muscle biopsies. EV RNA detection by molecular methods is now considered the gold standard for the diagnosis of enteroviral meningitis.

3. Ebola Virus Disease (EVD) or Ebola hemorrhagic fever

Ebola virus disease (EVD), formerly known as Ebola haemorrhagic fever, is a severe, often fatal illness in humans. The virus is transmitted to people from wild animals and spreads in the human population through human-to-human transmission. Relapse-symptomatic illness in someone who has recovered from EVD due to increased replication of the virus in a specific site is a rare event, but has been documented. It can be difficult to clinically distinguish EVD from other infectious diseases such as malaria, typhoid fever and meningitis. Careful consideration should be given to the selection of diagnostic tests, which take into account technical specifications, disease incidence and prevalence, and social and medical implications of test results. World Health Organization (WHO) recommended test include the detection of the virus from molecular level. This includes automated or semi-automated nucleic acid tests (NAT) for routine diagnostic management.

4. Human Immuno Deficiency Virus (HIV)

Even though HIV takes a tremendous toll each year, the population of people *living* with the disease is about 35 million. HIV diagnostic testing has come a long way since its inception in the early 1980s. Current enzyme immunoassays are sensitive enough to detect antibody as early as one to two weeks after infection. A variety of other assays are essential to confirm positive antibody screens (Western blot, polymerase chain reaction PCR), provide an adjunct to antibody

testing (p24 antigen, PCR), or provide additional information for the clinician treating HIV-positive patients (qualitative and quantitative PCR, and genotyping).

5. Infectious Diarrheal Disease

The majority of cases about 1.7 billion globally each year prevails. According to Carol Sulis, University of Columbia, there are many pathogens that can cause these infections, but the whole class of diseases categorized as infectious diarrhea is deadly. The best diagnostics and therapeutics for diarrheal diseases have been developed based on an understanding of the basic pathophysiology of the pathogens involved. Cholera, the prototype of secretory diarrhea, is caused by the enterotoxin of *Vibrio cholerae* (cholera toxin). Similar to *V. cholerae*, enterotoxigenic *E. coli* (ETEC, the major cause of traveler's diarrhea) produce enterotoxins that activate adenyl cyclase and guanyl cyclase, respectively, causing chloride secretion to the intestinal lumen. For many years, enteric infections have been diagnosed by analysis of bacterial cultures and microscopy to detect ova and parasites. Bacterial genes can be detected in stool samples using molecular diagnostic techniques, although this methodology is still limited to research settings. Many laboratories have replaced diagnostic methods, based on microscopy of fecal samples, with more sensitive and specific (and less observer-dependent) ELISA methods; ELISA is used to detect protozoa such as *Giardia* and *Cryptosporidium* in fecal samples. PCR analysis can detect most protozoan infections and is more sensitive than antibody detection methods although these assays are still not performed routinely in the clinic.

GENETIC DISEASE

Molecular genetic tests are defined as any analysis of genetic material that helps to establish diagnosis, choice of treatment, long term follow-up of a patient and family counseling. Some of the common genetic disorder where the molecular diagnosis plays crucial role are described below:

1. Cystic Fibrosis

Cystic fibrosis (CF) is a monogenetic disorder that presents as a multisystem disease. The first signs and symptoms typically occur in childhood, but nearly 4 percent of patients are diagnosed as adults. This disease is characterized by chronic airway infection that ultimately leads to bronchiectasis and bronchiolectasis, exocrine pancreatic insufficiency and intestinal dysfunction, abnormal sweat gland function, and urogenital dysfunction. Because of the large number of CF mutations, DNA analysis is not used for primary diagnosis. The diagnosis of CF rests on a

combination of clinical criteria and analyses of sweat Chlorine- values. A number of different screening strategies have been suggested, including prenatal, preconceptional, school prenatal screening has a practical advantage because of existing facilities, while with screening before conception all reproductive options are, in principle, open to detected
carrier couples. Prenatal diagnosis is primarily performed by a PCR analysis of samples obtained by CVS (Chorionic Villus Sampling), amniocentesis or percutaneous villus sampling.

2. Sickle Cell Disease

Sickle cell disease evolved as a genetic mutation in areas of the world where malaria is endemic. The gene responsible for the disease is located on the short arm of chromosome 11. It is inherited as an autosomal recessive inheritance in which the abnormal gene product is an altered Beta chain in the hemoglobin structure. The pathogenesis of the disease is hypothesized to be due to the adherence of sickle cells to vascular endothelium, which initiates and contributes to micro vascular occlusion and pain episodes. Screening programs for the detection of HbS gained popularity in the 1970s. The most useful test for the diagnosis of hemoglobinopathies includes hemoglobin electrophoresis for variant identification. Other methods include isoelectric focusing, high-performance liquid chromatography (HPLC) or DNA analysis.

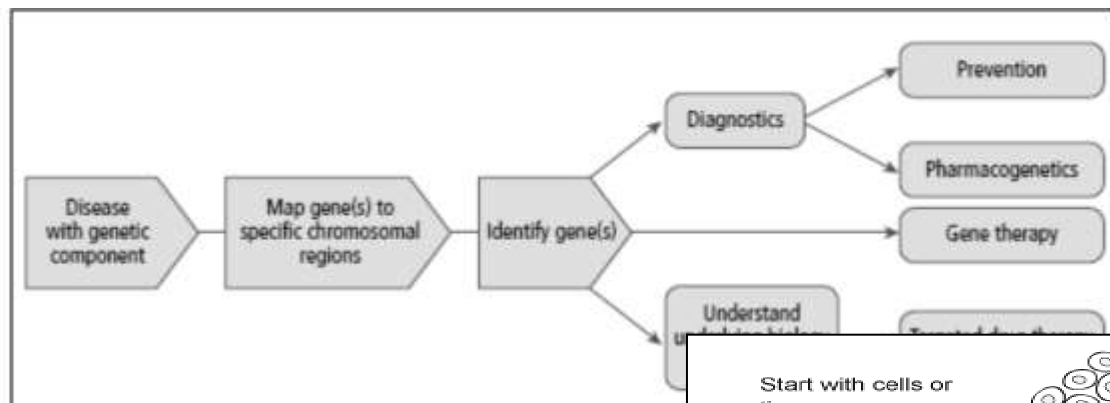
3. Huntington's disease

This is an inherited disease which causes certain nerve cells in the brain and central nervous system to degenerate. Loss of these nerve cells causes symptoms such as behavioral changes, unusual snake-like movements (chorea), uncontrolled movement, difficulty walking, loss of memory, speech and cognitive functions and difficulty in swallowing. Scientists identified the defective gene that causes Huntington's disease in 1993. A diagnostic genetic test is now available.The test can confirm that the defective gene for huntingtin protein is the cause of symptoms in people with suspected Huntington's disease and can detect the defective gene in people who don't yet have symptoms but are at risk because a parent has Huntington's.

CANCERS

Cancer can be defined as a disease in which a group of abnormal cells grow uncontrollably by disregarding the normal rules of cell division. Normal cells are constantly subject to signals that dictate whether the cell should divide, differentiate into another cell or die. Cancer cells develop a degree of autonomy from these signals, resulting in uncontrolled growth and proliferation. If this proliferation is allowed to continue and spread, it can be fatal. Phenomenal advances in

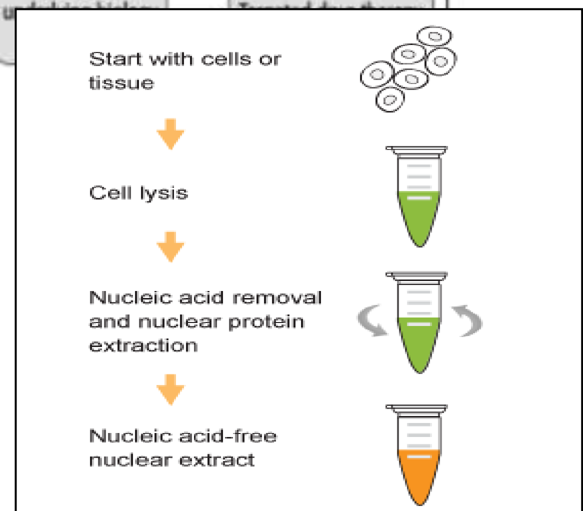
cancer research in the past 50 years have given us an insight into how cancer cells develop this autonomy. We now define cancer as a disease that involves changes or mutations in the cell genome. These changes (DNA mutations) produce proteins that disrupt the delicate cellular balance between cell division and quiescence, resulting in cells that keep dividing to form cancers. "Cancer biomarkers" constitutes one of the most rapidly advancing fields in clinical diagnostics. They can be used to screen asymptomatic individuals in the general population, to assist in early and specific diagnosis in suspect cases, to select patients who may benefit from specific treatments, to predict prognosis and response to therapy and finally to monitor patients after primary therapy. The introduction of advanced sophisticated technologies like microarray, mass spectrometry and automated DNA sequencing have opened new avenues in the field of "Cancer biomarkers". Conventional histopathology based on assessing morphology has remained the standard diagnostic method for many years. The use of enzyme histo-chemistry and electron microscopy expanded the primary micro-anatomic evaluation to include biochemical and sub-cellular ultra-structural features. More recently, the progress in immuno- histochemistry, cytogenetics, analysis of DNA ploidy and molecular genetic assays have been added as valuable adjuncts to light microscopy in cancer diagnosis.



GENERAL METHOD

1. Nucleic Acid Isolation

The first step of most molecular pathology tests is isolation of DNA or RNA from a patient specimen, by either manual or automated methods. Nucleic acid purification begins with lysis of the cells in the



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sample. Cell lysis liberates cellular macromolecules including proteins, lipids, and nucleic acids. Cell lysis can be accomplished using a detergent solution to break cell membranes and remove lipids. Proteins are enzymatically degraded with protease or selectively precipitated. Protein digestion is performed at about 56 °C which permanently denatures many proteins but does not affect nucleic acids. This process is followed by selective nucleic acid extraction that takes advantage of the physical and chemical differences between nucleic acids and other cellular molecules, forming the basis for their isolation. The nucleic acid
is then purified from the soluble contaminants produced in the extraction method by precipitation in an ethanol–salt solution. Nucleic acid (RNA/DNA) can be isolated via any one of the following methods:

a. Organic (Phenol) Extraction

Nucleic acids have a strong net negative charge because of the phosphate groups in the sugar–phosphate backbone, and thus are highly soluble in an aqueous environment. By contrast, proteins, lipids, and carbohydrates contain varying proportions of charged and uncharged domains producing hydrophobic and hydrophilic regions. This difference makes proteins entirely soluble in organic solutions or confines them to the interface between the organic and aqueous phases during an organic extraction. This characteristic forms the basis for phenol–chloroform extraction, in which phenol is added to an aqueous solution containing cellular constituents, mixed, and then centrifuged to separate the aqueous and organic phases. This method produces high-quality nucleic acids but is relatively labor-intensive, employs hazardous chemicals, and produces liquid organic waste.

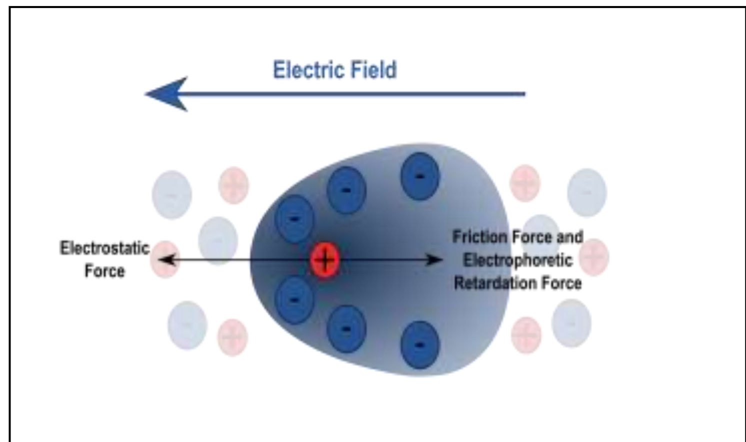
b. Ethanol–Salt Precipitation

Nucleic acids can be precipitated in an aqueous solution by the addition of concentrated ethanol and salt. Ethanol makes the solution hydrophobic, while salt increases the ionic strength of the solution, thereby reducing the repulsion of the negatively charged sugar–phosphate backbone of nucleic acids. Centrifugation allows the nucleic acid precipitate to be collected and resuspended in a dilute salt buffer (TE buffer).

c. Magnetic Bead Extraction

Another solid phase extraction method uses ligand-coated magnetic beads to capture nucleic acids. After cell lysis, DNA molecules are attracted to the ligands on the magnetic beads. The beads are immobilized by a magnet, allowing multiple washings of the bound nucleic acids to

remove proteins and other contaminants. The nucleic acids then are eluted from the ligands on the magnetic particles with an elution buffer.



2. Electrophoresis

In electrophoresis, an electric field is used to separate charged molecules by differential mobility in a sieving matrix that can be either liquid or solid (gel). The differential mobility is determined by the size of the molecule and its conformation, the net charge of the molecule (as modified by pH), temperature, and the pore size of the matrix. DNA, being negatively charged, migrates towards the anode (+) when an electric field is applied to an electrolyte solution. The size of DNA can be modified by restriction endonuclease digestion, rendering DNA fragments small enough to be mobile in the matrix. Nucleic acid conformation can be modified with denaturing conditions prior to or during electrophoresis. Nucleic acids usually are electrophoresed at a slightly alkaline pH to ionize all phosphate groups in the backbone of the molecule, enhancing the negative charge which allows nucleic acids to be moved in the electrophoretic field.

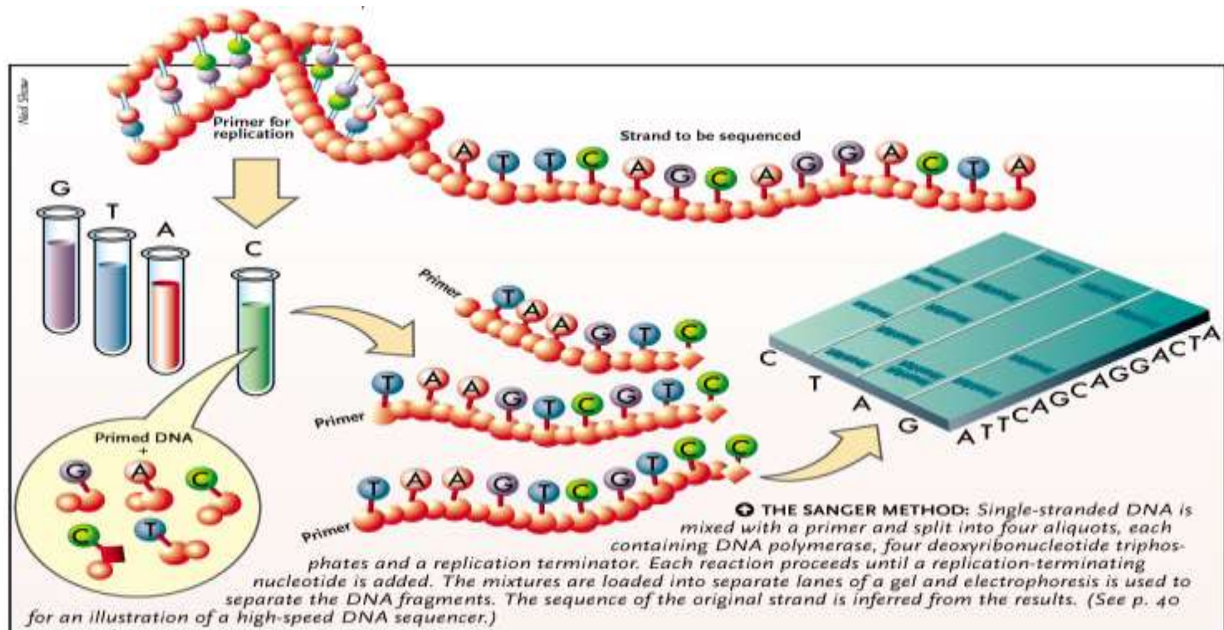
3. Restriction Endonucleases

Restriction endonuclease digestion is commonly used as a component of clinical molecular tests. Restriction endonucleases (REs) cleave DNA at specific nucleotide recognition sequences. Restriction endonucleases are naturally occurring proteins produced by and purified from bacteria. Each bacterial species contains one or more REs, each recognizing a unique sequence of base pairs in double stranded DNA, called recognition sites (most commonly 4–8 bp long). The natural function of REs within bacteria is to digest and inactivate foreign DNA (such as bacteriophage DNA). The frequency of recognition sites in target DNA for any given RE is inversely proportional to the size of the recognition site. Some REs do not cleave DNA when their recognition sites are methylated; this can be useful in certain clinical laboratory applications such as detection of imprinted genes in genetic diseases or promoter

hypermethylation in tumors. Some mutations occur at RE recognition sites and can be detected by a change in the RE digestion pattern of a PCR product or genomic DNA. Unique DNA restriction fragment patterns are generated by digestion with different REs, creating a range of DNA restriction fragment sizes, which can be fractionated and detected using agarose gel electrophoresis.

SPECIFIC METHOD

1. DNA Sequencing



THE SANGER METHOD: *Single-stranded DNA is mixed with a primer and split into four aliquots, each containing DNA polymerase, four deoxyribonucleotide triphosphates and a replication terminator. Each reaction proceeds until a replication-terminating nucleotide is added. The mixtures are loaded into separate lanes of a gel and electrophoresis is used to separate the DNA fragments. The sequence of the original strand is inferred from the results. (See p. 40 for an illustration of a high-speed DNA sequencer.)*

The ability to sequence DNA has been essential to the field of molecular pathology because sequence information is a prerequisite for PCR, PCR alternatives, and hybridization with probes necessary for successful Southern blot analysis. The method for DNA sequencing developed by Sanger, Nicklen, and Coulson is the basis for most DNA sequencing performed both in clinical laboratories. The Sanger sequencing reaction uses a single DNA primer and DNA polymerase resulting in linear, rather than the exponential, PCR amplification. Components essential to the Sanger sequencing reaction include: (1) DNA template that is purified and quantitated; (2) sequence-specific primers, complementary to the opposite strands and ends of the DNA region to be sequenced, which is desalted and usually purified by high-performance liquid

chromatography (HPLC); (3) small proportions of dideoxynucleoside triphosphates (ddNTPs) in addition to the conventional deoxyribonucleoside triphosphates (dNTPs) used in DNA sequencing reaction; and (4) an electrophoresis technique capable of clearly distinguishing single nucleotide length differences in DNA strands dozens or hundreds of nucleotides in length. Conventional DNA sequencing with polyacrylamide gel electrophoresis (whether using manual or automated sequence detection) is time-consuming and labor-intensive. The introduction of CE facilitated the use of sequencing and fragment analysis by the clinical laboratory

Examples of Applications of DNA Sequencing

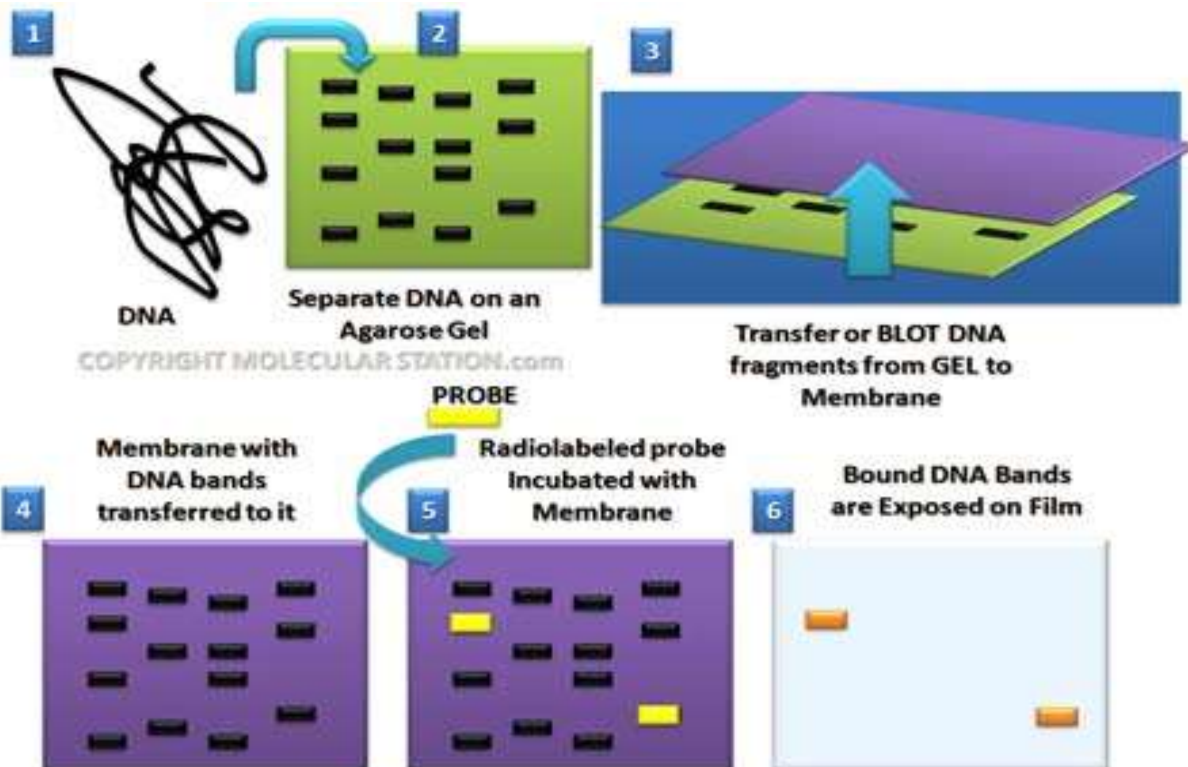
a. *CFTR* mutation analysis for cystic fibrosis

b. *BRCA1* mutation analysis for breast/ovarian cancer

c. *CEBPA* mutation analysis for acute myeloid leukemia
(AML)

d. High-resolution human leukocyte antigen (HLA) typing for allogeneic bone marrow transplantation

2. Southern Blot



The Southern blot was developed by E.M. Southern in 1975 and was the first molecular biology

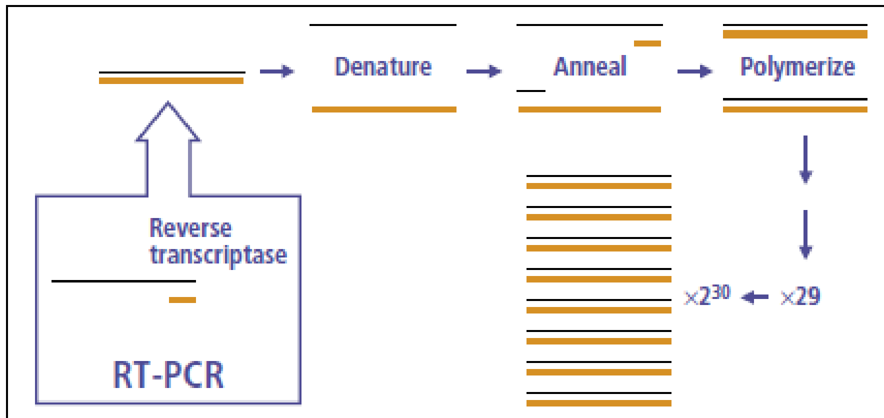
tool to have a major impact on clinical molecular pathology. The Southern blot is in limited use, having been largely replaced by amplification methods. Development of the Southern blotting was based on prior knowledge of nucleic acid isolation, gel electrophoresis, restriction endonuclease digestion, and nucleic acid probe labeling for detection of DNA sequences of interest. "Blotting" is the transfer of the fractionated DNA from the gel to a solid support such as a nylon membrane. Because no amplification of target DNA occurs, Southern blot analysis requires a large mass of DNA. Because the banding pattern depends on the specific cuts made by the restriction endonuclease and not just random DNA breaks, the DNA must be largely intact and of high molecular weight.

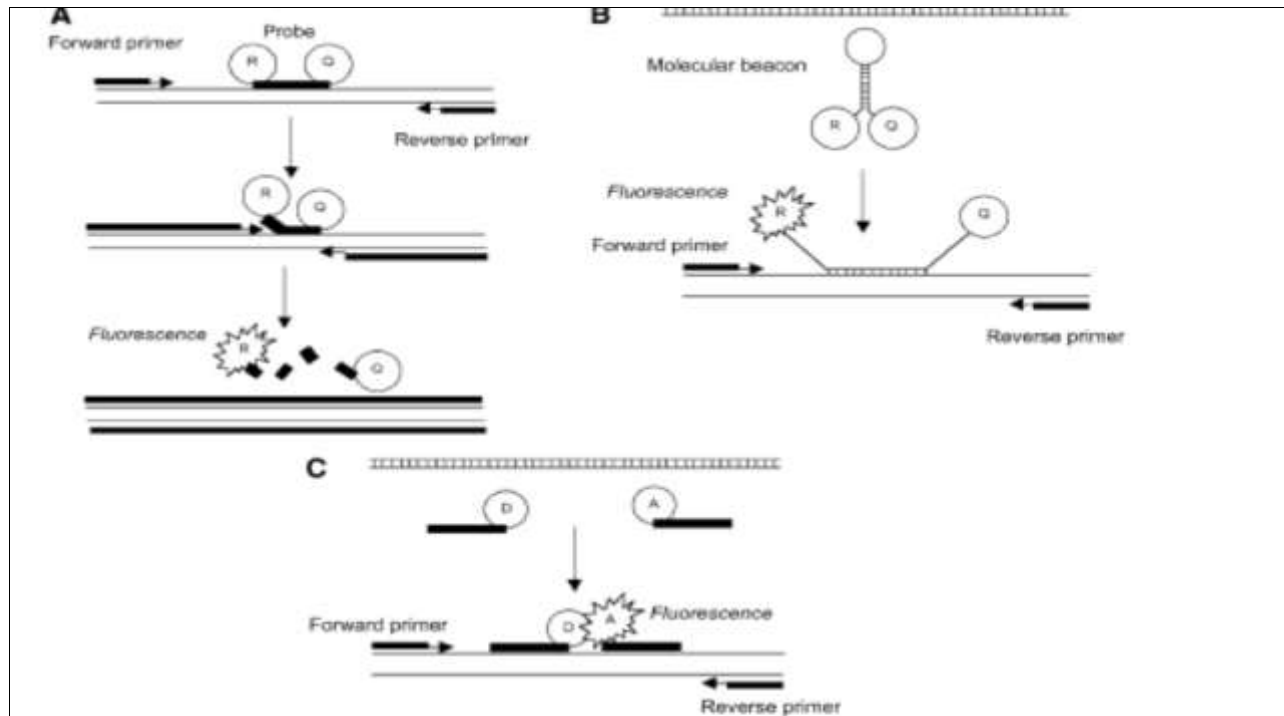
Applications of Southern Blot

a. Fragile X syndrome diagnosis

b. Myotonic dystrophy diagnosis

3. Polymerase Chain Reaction





In the mid-1980s, Mullis and coworkers developed a method, the polymerase chain reaction (PCR), to amplify target sequences of DNA exponentially. As the name suggests, the method is a DNA polymerase-mediated cyclical reaction resulting in amplification of specific nucleic acid sequences. Arguably, PCR is the single most important "invention" leading to the development of a new discipline in clinical laboratory medicine, that is, molecular pathology. Both PCR and the Southern blot are techniques used to investigate specific genomic targets. However, PCR is orders of magnitude more sensitive and rapid, permitting turnaround times from specimen receipt to report generation of 24 hours or less. PCR lends itself to much higher test volumes than Southern blotting, a crucial point in its adoption in the clinical laboratory setting. Opportunity for high test volumes, excellent specificity and sensitivity, and the rapid turnaround times of PCR are the principal reasons this technology is used so widely in clinical molecular laboratories.

Examples of Application of PCR and its Variations

a. Identification of mutations in the *CTFR* gene in cystic fibrosis

b. Identification of mutations in the *ATM* gene in ataxia–telangiectasis

c. Detection of enterovirus and herpes simplex virus (HSV) nucleic acids in cerebrospinal fluid (CSF)

d. Detection of pathogenic enteric bacteria in stool

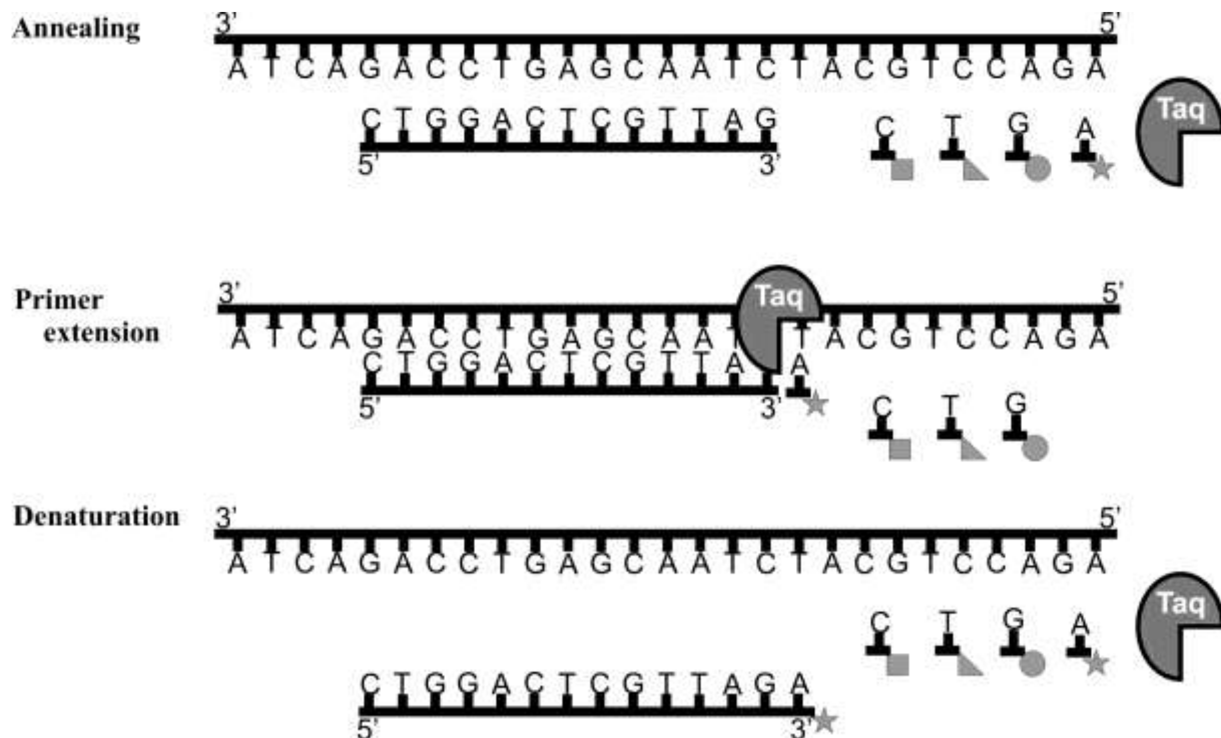
e. Analysis of multiple *BRCA1* loci in a breast cancer patient

f. Identification of different bacteria in a respiratory infection specimen

g. Amplification of multiple microsatellite loci for bone marrow engraftment analysis

4. Single Nucleotide Extension

Another method for a multiplexed assay is single nucleotide extension (SNE) or single base extension (SBE). In this method, either a single long-range PCR or a multiplexed PC is used to amplify the region(s) of interest. This is followed by a multiplexed set of extension primers of differing lengths that hybridize one base upstream to the variant(s) of interest. A second, linear amplification, similar to Sanger sequencing, adds the next nucleotide (at the variant position) using ddNTPs, with each type labeled with a different fluorophore. The products are separated by capillary electrophoresis or mass spectrometry, and the specific fluorescent signal of the incorporated base indicates which base was added, and whether the variant is present or not. This method can be used to genotype up to approximately 20 mutations at once.



<u>Examples of Applications of Single Nucleotide Extension</u>

a. Analysis of common mutations in *GALT* for galactosemia

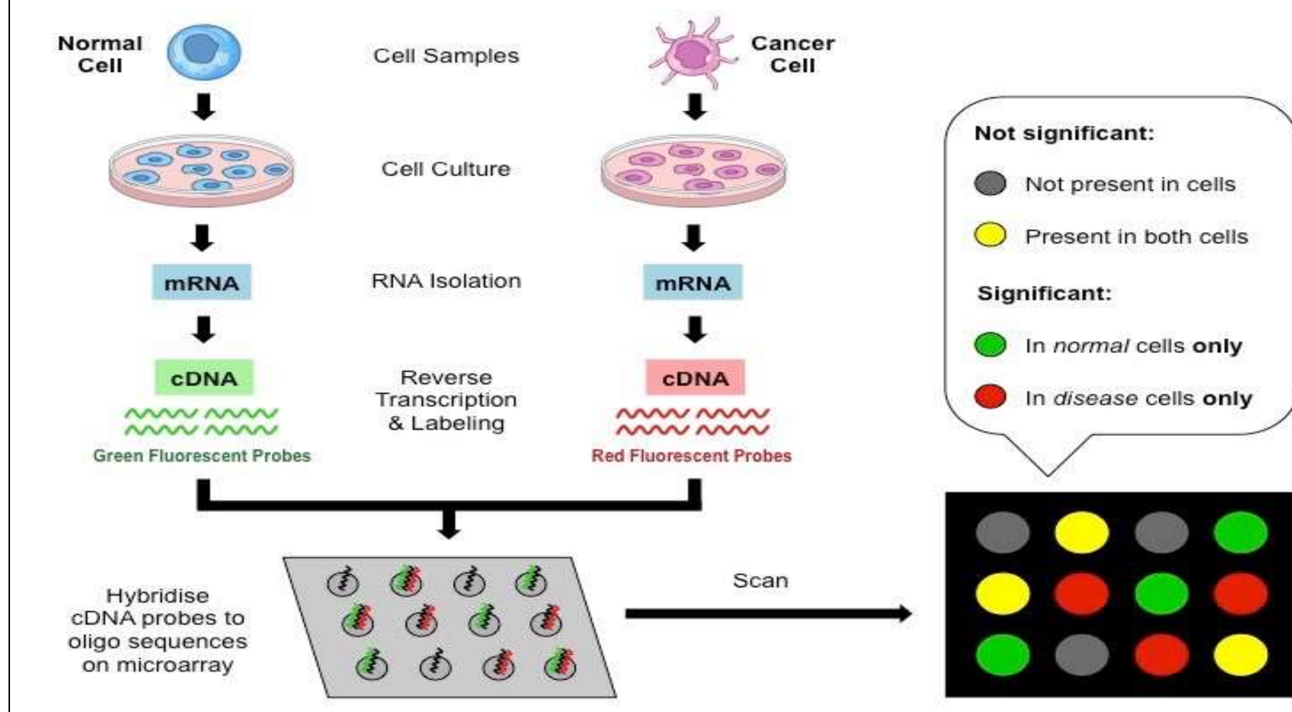
b. Analysis of common mutations in *BTD* for biotinidase deficiency

3. Analysis of multiple mutations in the *CFTR* gene for cystic fibrosis

5. DNA microarrays

Gene expression profiling using DNA microarrays holds great promise for the future of molecular diagnostics. This technology allows, in one assay, for simultaneous assessment of the expression rate of thousands of genes in a particular sample. The 2 types of DNA microarrays that are widely used are cDNA microarrays and oligonucleotide/DNA chips. In cDNA microarrays, DNA sequences complementary to a library of mRNA from thousands of genes are mechanically placed on a single glass slide. The immobilized cDNA sequences serve as anchoring probes to which mRNA extracted from the tested sample will specifically attach during hybridization. If the tested mRNA is first tagged with a fluorescent dye, the intensity of fluorescence at each anchoring probe location would be proportional to the amount of mRNA (degree of expression) of the gene at that location. A microarray reader displays the intensity of fluorescence at each cDNA location as a colored dot per gene location on a grid.

Oligonucleotide/DNA chips are silicon chips on which the "anchoring" oligonucleotide sequences are directly synthesized and serve as the immobilized probes to which the complementary specific mRNA will hybridize. DNA chips can be made with an astonishing density of gene arrays encompassing up to 12,000 or more genes on a single chip.



CONCLUSION

The molecular methods used in the clinical laboratory will continue to evolve and develop as researchers and instrument manufacturers develop new methods that improve on the sensitivity, specificity, cost and speed of current methods. Drivers for adoption of new technologies in the clinical laboratory include but are not limited to reduced technologist hands-on time, reduced cost, shortened turnaround time, interfacing of results to information systems to reduce human transcription errors, and improvements in the detection of analytes with clinical significance. Molecular pathology will continue to be at the leading edge of methods development, resulting in a constant stream of new test and method validations with all the required steps new tests require from a regulatory compliance perspective. While this is challenging for the molecular pathology laboratory leadership and staff, this innovation also is one of the exciting aspects of molecular pathology practice.

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