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Review Article

# Diagnostic techniques in molecular biology – an overview

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## Abstract

The developments of nucleic acid-based testing have been necessitated by newer approaches to therapy and disease diagnosis through techniques involving genetic analysis. This has been achieved by the introduction of molecular diagnostic techniques that target the biosynthesis interactions between specific biomolecules such as DNA, RNA and proteins. These molecular diagnostic techniques include molecular cloning, macromolecule blotting and probing, gel electrophoresis, polymerase chain reaction, fluorescent in situ hybridization, spectral karyotyping imaging and DNA microarrays. These techniques have been applied in cases of prenatal tests, and diagnoses of infectious diseases and cancer. In situations of disease risk management, modern techniques in clinical diagnosis including molecular techniques have been applied. However, molecular diagnostics are rapid but are very expensive to install, hence the costs of health care should be considered alongside their potential advantages. Also, well-controlled outcome studies have been essential in demonstrating the efficacy of these technologies. In conclusion, the application of molecular diagnostics has advanced the practice of medicine with enhanced management and follow-up studies of comparative clinical (medical and surgical) cases.

**Keywords:** Molecular cloning, gel electrophoresis, polymerase chain reaction, fluorescent in sutu hybridization, spectral karyotyping imaging, DNA microarrays.

## Introduction

The application of nucleic acid-based testing to disease diagnosis and therapy at high accuracy and reduced cost offers revolutionary progress in human and animal genomics and this has altered the fundamental of medicine [37,58,77]. Previously, the analysis of deoxyribonucleic acid (DNA) by biochemists was difficult due to its minute structure until the early 1970s [2,79]. Also, the examination of nucleotides sequences that formed the genetic material of organism was only possible indirectly through protein or ribonucleic acid (RNA) sequencing [23,124] or by genetic analysis [95]. However, other approaches involving the direct analysis of DNA [95], isolation of specific regions of genomes and conscious manipulation of genes in genetic engineering [40,118] and recombinant DNA technology [42,74] have been developed.

Molecular biology is the molecular basis of biological activity between biomolecules in the various cellular systems of the body. Biological activities in the body include biosynthesis of DNA, RNA and proteins, the interactions between these molecules and the regulations of their interactions [42,63]. Molecular diagnostics on the other hand are collections of techniques used in the analysis of biological markers in the genome and proteome by applying molecular biology to medical testing [84,103]. These techniques have been reported to be vital in the diagnosis of inherited genetic diseases such as cystic fibrosis [92] and haemochromatosis [83], infectious diseases [18], oncology [111], leukocyte antigen typing (investigation and prediction of immune function) [22,33,64] and coagulopathies [10] as well as in pharmacogenomics (the genetic prediction of which drugs will work best) [81]. Molecular diagnostics therefore provides relevant preliminary information for the successful application of gene therapy [21,58], biologic response modifiers [76,40], the assessment of disease prognosis [52] and therapy response as well as detection of minimal residual disease [73]. Hence, this paper provides a summary of the history, various types and applications of commonly utilized molecular diagnostic techniques in biological sciences.

## Brief History of Molecular Diagnostic Techniques

The field of molecular biology and its clinical application grew in the late twentieth century [59,42]. In 1980, prenatal genetic test for Thalassemia was suggested and this test relied on restriction enzymes (endonucleases such as BamHI) that cut DNA. This test allowed for the

recognition of specific short sequences, created by different lengths of DNA strands dependent on the allele (variant form of the gene) [55]. In the 1980s, phrases used in the names of companies involved in developing these techniques included Molecular Diagnostics Incorporated [25], Bethseda Research Laboratories Molecular Diagnostics [57,82] etc.

In the 1990s, a distinct field of molecular and genomic laboratory medicine was developed following the identification of newly discovered genes and new techniques for DNA sequencing [7,50,117,71,72,31]. In 1995, the Association for Molecular Pathology (AMP) was formed to follow up on the new discoveries which led to establishment of The Journal of Medical Diagnostics in 1999 [31]. The Expert Reviews in The Journal of Medical Diagnostics was launched in 2001 by Informa Healthcare [84]. Later in 2002, information regarding recurrence of one-letter genetic differences (the single nucleotide polymorphisms) in human population as well as their relationship with the disease was accumulated and published by the HapMap Project [42]. In 2012, molecular diagnostic techniques for Thalassemia use genetic hybridization tests to identify the specific single nucleotide polymorphism causing an individual's disease [6].

However, the importance of commercial application of molecular diagnostics has created debate about patenting of the genetic discoveries at its heart [59]. In 1998, the European Union's Directive 98/44/EC clarified that patents on DNA sequences were allowable [90]. In 2010 in the US, AMP sued Myriad Genetics to challenge the latter's patents regarding two genes, BRCA1and BRCA2, which are associated with breast cancer [13]. In 2013, the U.S. Supreme Court partially agreed, ruling that a naturally occurring gene sequence could not be patented [86]. With advancement in molecular diagnostics, the detection of specific nucleotide sequences in DNA and RNA related or unrelated to disease has been made possible [31]. These nucleotide sequences may be due to changes such as gene rearrangements, insertion and deletion [71,72,90]. This therefore has led to the efficiency, accuracy and rapid growth in diagnostic with accentuation of personalized therapy [94].

## Molecular Diagnostic Techniques

### Molecular Cloning

In molecular biology, molecular cloning has been used as a basic tool to highlight the functions of proteins (Souii et al., 2013) [94]. In this technique, DNA coding for a protein of interest is cloned (using PCR and/or restriction enzymes) into a plasmid (known as an expression vector) [113,5]. A vector has 3 distinctive features: an origin of replication, a multiple cloning site (MCS), and a selective marker (usually antibiotic resistance) [26,5]. The origin of replication has promoter regions upstream from the replication/transcription start site [125,5].

This plasmid can be inserted into either bacterial or animal cells [113]. Introducing DNA into bacterial cells can be done by transformation (via uptake of naked DNA) [30], conjugation (via cell-cell contact) [27] or by transduction (via viral vector) [48]. Introducing DNA into eukaryotic cells, such as animal cells, by physical or chemical means is called transfection [41,121]. Several transfection techniques are available and these include calcium phosphate transfection, electroporation, microinjection and liposome transfection. DNA can also be introduced into eukaryotic cells using viruses or bacteria (Agrobacterium tumefaciens) as carriers; the latter is sometimes called bactofection [113]. The plasmid may be integrated into the genome, resulting in a stable transfection, or may remain independent of the genome and this is referred to as transient transfection [108]. In either case, DNA coding for a protein of interest is inside the cell, and the protein can be expressed [120,41].

In molecular cloning, a variety of systems including inducible promoters and specific cell-signaling factors, are available to help express the protein of interest at high levels [113]. Large quantities of a protein can then be extracted from the bacterial or eukaryotic cell [15]. Following extraction is the test for enzymatic activities after which the protein is crystallized to study its tertiary structure as well as the activity of new drugs against it [29].

### Macromolecule Blotting and Probing

"Blotting," is a term that refers to the process of detecting the presence and quantity of DNA, RNA, or protein in cells [100]. The blotting and probing techniques were first described by Edwin Southern (1973) for the hybridisation of blotted DNA [100]. In 1984, Patricia Thomas developed the RNA blot and this became known as the northern blot [98]. Further modifications and combinations of these protocols gave rise to other techniques such as southwesterns (protein-DNA hybridizations), northwesterns (to detect protein-RNA interactions) and farwesterns (protein-protein interactions), as reported in literature [98].

***Northern blotting:*** In the northern blotting, the structure and quantity of RNA are emphasized in relation to their expression patterns among different samples of RNA [107]. It is one of the most basic tools for determining the time, levels and conditions certain genes are expressed in living tissues [25]. The protocol involves using a combination of denaturing RNA gel electrophoresis and

a blot [107]. In this technique, RNAs are separated based on size. The separated RNAs are then transferred to a membrane probed with a labeled complement of a sequence of interest [110]. The results visualized in the establishment of bands represent the sizes of the RNA detected while the intensity of these bands is related to the amount of the target RNA in the samples analyzed [107]. The procedure is commonly used to study when and how much gene expression is occurring by measuring the quantity of RNAs present in different samples [107,110]. The major disadvantages of the northern blot technique were its poor sensitivity and high time consumption due to the use of the traditional DNA oligonucleotide probes [110]. These have been overcome by adoption of an improvised protocol of miRNA analysis involving RNA extraction, polyacrylamide gel electrophoresis with northern blotting, and the detection of locked nucleic acid (LNA)-modified oligonucleotide probes by hybridization [110].

***Western blotting:*** In western blotting, the detection of proteins is first carried out followed by separation based on size and molecular weight using a thin gel sandwiched between two glass plates [115]. This technique is referred to as sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [115,2,8]. The proteins in the gel are then transferred to a support membrane probed with solutions of enzymes-labeled antibodies [115]. These support membranes include polyvinylidene fluoride (PVDF), nitrocellulose, nylon e.t.c [8]. The specificity of antibodies-protein binding is visualized by a colored product (chemiluminescence) or autoradiography [115]. However, the use of western blotting techniques allows for not only detection but also quantitative analysis [2,56,8].

***Eastern blotting:*** The Eastern blotting technique is a modification of western blot involving the enzymatic detection of post-translational proteins [98]. The proteins blotted on to the PVDF or nitrocellulose membranes are probed for modifications using specific substrates [9].

**Gel Electrophoresis**

Gel electrophoresis is one of the basic tools of molecular biology [115]. The term "electrophoresis" was originally meant to refer to the migration of charged molecular particles in an electrical field, especially across a membrane [17]. However, the migration of lower molecular weight substances in stabilized media such as gels and powders has been referred to as "ionophoresis" [99]. The basic principle is that by means of an electric field and size, DNA, RNA, and proteins can all be separated (Weber and Osborn, 1969) [115]. In agarose gel electrophoresis, DNA and RNA are separated on the basis of size by running the substances through an electrically charged agarose gel [43,17].

**Polymerase Chain Reaction**

Polymerase Chain Reaction (PCR) is a revolutionary method developed in 1983 by Kary Mullis (Kellenberger, 2004) [59]. PCR has proved to be a valuable method and remained the most frequently used molecular technique in molecular pathology laboratories [73] and it is an extremely versatile technique for copying DNA with the aid of DNA polymerase [37,24,75]. In this technique, the predetermined copying or modification of a specific DNA sequence and identification of particular DNA fragment in a cDNA library is enhanced by DNA polymerase enzyme, which amplifies the specific fragments of the target DNA molecule added to the reaction [24,19]. These nucleotides are named as primers and contain the sequences complementary to the target sequences of the target DNA molecule [2]. The PCR technique can also be used to introduce restriction enzyme sites to ends of DNA molecules, or to change particular bases of DNA (referred to as site-directed mutagenesis) [43]. The sequence of reactions in PCR is extremely powerful such that amplification of a DNA molecule produces about 1 billion molecules under a short period of time (less than 2 hours) as the reaction is done under perfect conditions [2,24].

PCR has variations such as reverse transcription PCR (RT-PCR) for amplification of RNA and quantitative PCR which allow for quantitative measurement of DNA or RNA molecules [24]. Multiple copies of a targeted chimeric gene can be obtained by using a pair of priming complementary sequences (oligonucleotide primers) together with unique heat-resistant polymerases (DNA copying enzymes) [35]. The Multiplex PCR (mPCR) which is employed for the simultaneous identification of several gene sequences belonging to the same pathogen or originating from a mixture of different pathogens [106].

Each PCR cycle involves 3 basic steps: denaturing, annealing (or hybridization), and polymerization [43,24]. During denaturing, the 2 strands of the helix of the target genetic (DNA) material are unwound and separated by heating at 90° to 95°C. During annealing, there is binding of oligonucleotide primers to their complementary bases on the single-stranded DNA. This step requires a much cooler temperature, 55°C. Finally, during polymerization (at 72 to 75°C), the template strand is read by polymerase and is paired rapidly with the appropriate nucleotides, resulting in 2 new helices consisting of part of the original strand and the complementary strand that was just assembled [24,75]. The process is repeated 30 to 40 times, with doubling of the amount of the targeted

genetic material in each cycle. At the end of the procedure, multiple identical copies (in millions) of the original specific DNA sequence would be produced. The copies are expected to migrate concurrently when subjected to electrophoresis to form a single band due to their similarities in electrical charge and molecular weight [53,24].

The specificity of a PCR assay is determined by the target DNA sequence under evaluation, the sequence of the oligonucleotide probe, similar sequences that may exist elsewhere in nature and the intentions of the assay designer [35]. Alternative amplification methods in existence include Loop Mediated Isothermal Amplification (LAMP) [101], Low-Stringency Single-Specific-Primer (LSSCP) (Mohamed, 2012), Ligase Detection Reaction (LDR) [20], Ligase Chain Reaction (LCR) [116] and Single Stand Conformation Polymorphism (SSCP) [60].

### *Fluorescent in Situ Hybridization*

Fluorescent In Situ Hybridization (FISH) was developed by biomedical researchers in the 1980s, and is based on the use of fluorescence-labeled oligonucleotide probes [37,75] that specifically attach to their complementary DNA sequence target on the genome and the region is labeled with fluorescence color (e.g., Texas red, FITCI green, acridine orange) [61,35]. The labeled region can then be visualized under a fluorescence microscope (Amann and Fuchs, 2008) [3]. There are 3 types of probes in wide use:

- Painting probes: through the attaching of the painting probe to overlapping sequences on target chromosome (e.g., chromosome 17), the chromosome is identified as "painting" based on the chosen fluorescence color [61,60].
- Centromeric probes that identify the centromeric region of a specific chromosome and thus help in enumerating the number of copies of that chromosome even in a non-dividing cell interphase state) [61].
- Allele-specific probes that adhere to a specific target allele sequence such as the p53 tumor suppressor gene or the HER2/neu oncogene (George et al., 2003; Gary, 2007; Bernasconi, 2008) [37,35,12].

FISH offers great advantages over conventional cytogenetics in the study of chromosomal deletions and translocations, and gene amplifications [66,67,103]. Conventional cytogenetics requires a time-consuming cell culture step and can be performed only with fresh tissue samples (Trask, 1991) [104]. FISH is fast and sensitive and could be used as a complementary tool in genetic diagnostics as it can be performed on cells in dividing (metaphase) and resting (interphase) stages, fresh frozen tissues as well as archival cytologic smears or paraffin-embedded tissue sections [37,46,103]. This also allows FISH to be utilized in the differentiation of signals from cells in healthy and cancerous conditions as well as in the enhancement of "interphase cytogenetics" in both tumor and prenatal settings [105,3].

FISH is often used in interpretation of numerical and complex chromosome aberrations and the evaluation of HER2/neu oncogene amplification in breast carcinoma and for detection of different translocations in chronic myelogenous leukemia and acute myelogenous leukemia [35,80,103].

### Spectral Karyotyping Imaging

Spectral Karyotyping Imaging (SKI) is a cytogenetic technique, developed by [89], and it combined the two basic principles of FISH which are chromosome painting and multicolor fluorescence [105,80]. This involves the use of 24 sets of chromosome-specific "painting" probes [11,36,39]. SKI is based on the labeling of each probe with varying proportions of five fluorochromes, differently combined for each specific chromosome in a light of unique spectral emission [37]. This enables the display and identification of all 24 human chromosomes [47] assigned in different colours in a single metaphase, by using a combination of probe labelling, fluorescence microscopy, spectroscopy, CCD-imaging and spectral image analysis without prior knowledge of abnormalities involved [3,36,39].

This technology allows the use of an "interferometer" similar to those used by astronomers for differentiating light spectra emitted by different stars [37]. The slight variations in color, undetectable by the human eye, are detected by this computerized device. This then reassigns an easy-to-distinguish visual color (classification color) to each pair of chromosomes [36]. The benefits of this approach include the accurate analysis of abnormal karyotypes (numerical chromosome abnormalities) [62,39], unresolved by conventional cytogenetics and the ability to identify cryptic translocations in apparently 'normal' karyotypes (shifting in colored chromosomal portions) [36,47]. SKI is also used on dividing cells in metaphase thus, complementing conventional cytogenetics [114,39,49]. Furthermore, complex translocations occurring in tumor cells (e.g., breast cancer cells) can be resolved by SKI, leading to analysis of "marker chromosomes" composed of an amalgam of fragments from different chromosomes [36].

Despite the analytical importance of SKI, the following

limitations have been reported:

- Structural abnormalities, such as inversion, deletion, insertion, and duplication in the same chromosome are shown with the same color thus, impossible to evaluate [36].
- Also, the Q-positive segment and the satellite region of the long arm of the Y chromosome near the centromere cannot be detected [36].
- The resolution limit of detection is approximately 1-2 Mb, similar to conventional chromosome painting techniques, and minor structural abnormalities of less than 1 band cannot be detected [11]. Therefore, verification of chromosomal breakage site requires the use of SKI beforehand in combination with G-banding or high-resolution chromosome banding, instead of using SKI alone to macroscopically observe the banding patterns [119].

Moreover, the development of spectral color banding technique has overcome the limitations of SKI [54,51] and this technique combine G-band differential staining with the SKI coloring technology. The widespread clinical use of SKI in the field of clinical genetics has made significant contributions in molecular diagnosis of disorders but the cost of this technique still remains a drawback [36].

**DNA Microarrays**

Microarray refers to a small, two-dimensional high density matrix of DNA fragments which are printed or synthesized on a glass or silicon slide (chip) in a specific order [106]. DNA microarrays can be utilized for gene expression and simultaneous assessment of the expression rate of multiple genes in a particular sample [88,93]. The 2 types of DNA microarrays that are widely used are cDNA microarrays and oligonucleotide/DNA chips [34,38,106].

In cDNA microarrays, DNA sequences complementary to arrays of mRNA from multiple genes are mechanically placed on a single glass slide [51]. This is followed by specific attachment of the immobilized cDNA sequences serving as anchoring probes to which mRNA are extracted from the tested sample during hybridization [88]. The tagging of the tested mRNA with a fluorescent dye produces fluorescence at each anchoring probe location, the intensity of which is proportional to the amount of mRNA (expression degree) of the gene at that location [65]. A microarray reader normally displays the intensity of fluorescence at each cDNA location as a colored dot per gene location on a grid [37]. This computerized reader is linked to a database that indicates the gene at each intercept location [112].

Oligonucleotide/DNA chips comprise silicon chips on which the "anchoring" oligonucleotide sequences are directly synthesized [88]. This silicon chips serve as the immobilized probes to which the complementary specific mRNA will hybridize. DNA chips can be produced with large density of gene arrays encoding up to 12,000 or more genes on a single chip [37].

The DNA microarrays technique has been utilized in the analysis and comparison of numerous tumor samples through the building of gene expression "fingerprints" databases and linking of specific patterns of expression to primary site of origin, prognosis and outcome of therapy [85,93].

## Applications of Molecular Diagnostic Techniques

**Prenatal Tests**

Conventional prenatal tests involve the analysis of the number and appearance of chromosomes (the karyotype) [1]. Noninvasive prenatal testing using fetal DNA in maternal plasma has been adopted due to the presence of cell-free DNA in plasma [122]. The occurrence of mutation and inheritance patterns of diseases has given rise to prenatal diagnosis by use of direct or indirect methods of detection [97]. In direct mutation analysis, detection is highly accurate whereas in indirect mutation analysis, accuracy is dependent on the distance between DNA marker and disease locus (Tantravahi and Wheeler, 2003) [97]. This has been employed for chromosomal abnormalities such as Down Syndrome [1].

**Infectious Diseases**

Molecular diagnostics are used to identify infectious diseases such as chlamydia [102], influenza virus [28] and tuberculosis [78] or specific strains such as H1N1 virus [16]. FISH in combination with flow cytometry has been used for rapid culture independent detection of Salmonella spp. and in combination with PCR has been used for the accurate detection of Staphylococcus and Listeria spp. [106]. Genetic identification of infectious agents can be achieved as indicated in the diagnoses of malaria parasite by use of a loop-mediated isothermal amplification test [44]. However, despite these advances in genome analysis, infections are still more often identified by means of proteome, bacteriophage, or chromatographic profile [96]. Molecular diagnostics are also used to understand the specific strain of pathogens through the detection of drug resistance genes [96].

**Disease Risk Management**

A patient's genome may include an inherited or random mutation which affects the probability of developing a disease in the future [70]. For example, Lynch syndrome is a genetic disease that predisposes patients to colorectal and other cancers; early detection can lead to close monitoring that improves the patients' chances of a good outcome [109]. Cardiovascular risk is indicated by biological markers and screening can measure the risk that a child will be born with a genetic disease such as Cystic fibrosis [91]. Genetic testing is ethically complex: patients may not want the stress of knowing their risk [4]. In countries without universal healthcare, a known risk may raise insurance premiums [45].

Ionizing Radiation (IR) induces numerous stable and unstable chromosomal aberrations. Unstable aberrations, where chromosome morphology is substantially compromised, can easily be identified by conventional chromosome staining techniques. FISH probes can be specific for whole chromosome(s) or precise sub-region on chromosome(s) [103]. The method not only allows visualization of stable aberrations, but it can also allow detection of the chromosome(s) or specific DNA sequence(s) involved in a particular aberration formation [80]. Two highly sensitive cytogenetics methods to identify inter-chromosomal stable aberrations that form in the bone marrow cells after exposure to total body irradiation are the multiple fluorescence in situ hybridization (mFISH) and spectral karyotyping (SKY). Although both techniques rely on fluorescent labeled DNA probes, the method of detection and the process of image acquisition of the fluorescent signals are different. The use of these two techniques have been adopted in research areas including radiation biology, cancer cytogenetics, retrospective radiation biodosimetry, clinical cytogenetics, evolutionary cytogenetics, and comparative cytogenetics [80]. Application of variable FISH techniques have been found to enhance the thorough interpretation of numerical and complex chromosome aberrations, bridging the gap between conventional chromosome banding analysis and molecular genetic DNA studies of risk factors [103].

## Cancer

Cancer is a change in the cellular processes that cause a tumour to grow out of control [70]. Cancerous cells sometimes have mutations in oncogenes, such as KRAS and CTNNB1 (β-catenin) [68]. Analysing the molecular signature of cancerous cells (the DNA and its levels of expression via mRNA) enables physicians to characterize the cancer and to choose the best therapy for their patients [70]. In 2010, the incorporation of antibodies against specific protein marker molecules was developed and this could pave way for the development of multiplex assays that could measure many markers at a time [14]. Other biomarkers expressed in excessive nature in cancerous cells relative to in healthy ones include micro RNA molecules healthy ones [32]. The development of Molecular Diagnostics by Gliomas using next generation sequencing of a Glioma-Tailored Gene Panel has proved promising [123]. Expression levels from a collection of DNA samples can be used in predicting cancer. This is due to the vast number of genes expression level [93]. DNA microarray with the help of multiresolution analysis tool, Dual Tree M-Band Wavelet Transform (DTMBWT) for extraction at the 2nd level of decomposition and K-Nearest Neighbor (KNN) classifier, cancer classification into five different cancer datasets; Breast, Colon, Ovarian, CNS, and Leukemia with over 90% accuracy is now possible [93].

## Drug Development

The utilization of DNA barcoding, microarray technology and sequencing for the elucidation of plant genetic diversity and conservation has proved promising in molecular biology [74]. They are proving to be useful in authenticating the medicinal plants for herbal drug preparations [87]. This has the potential not only to classify the known and yet unknown species but also has a promising future to link the medicinally important plants according to their known and unnoticed properties in a considerably less time than usual [87,80]. The newer trends utilized in DNA chips and barcoding have paved the way for a future with many different possibilities. This can assist us to cure many different diseases and will also generate novel opportunities in medicinal drug delivery and targeting [87].

## Conclusion

Molecular diagnostics are changing every aspects of biological sciences. However, for each of these technologies, the additions to health care costs must be weighed against the potential advantages of more rapid diagnostics. The carrying out of well-controlled outcome studies are necessary to demonstrate the efficacy of these technologies. Furthermore, the classifications of neoplastic diseases by newer molecular techniques are expected to soon complement the currently familiar histology-based classification systems.

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