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Basic molecular techniques used in animal genetics

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Abstract
DNA the genetic material is unique to every organism. Identification of individual, detection of cause for diseases, in the field of selection of animals for breeding etc., evaluation of individual is very important and genotypic evaluation gives us accurate results. Therefore, in genetics molecular techniques and its utility has raised tremendously. This paper is written with an objective to through light on readers about few basic molecular techniques like DNA isolation, PCR, Agarose gel electrophoresis, Restriction enzymes, Cloning, recombinant DNA technology, hybridization, blotting and transgenic operations in a single go. The mechanism of action, principles of techniques, pros and cons of basic molecular technique along with its utility is detailed in this article.

Keywords: DNA, PCR, molecular techniques, utility

Introduction
Central dogma of life, DNA replication, Transcription and Translation all play a cornerstone of genetics (fig.1). Molecular genetics research using basing molecular techniques has made new area of possibilities in disease diagnosis. Whereas, advanced technology in molecular biology has become an important tool for detection of novel genes playing important role in the field of medicine, health of living being, production of livestock, farm production, environment and other related areas [1].

Molecular Genetics is a field of biology that investigates how differences in DNA molecule expression or structure lead to animal diversity. Screening of genetic material is always used in molecular studies in genetics "investigate" the function and/or structure of the genes in genome of an organism.

The genetic code of all organisms are made up of adenine, guanine, cytosine and uracil, combination of these base pairs when read in triplicate produce different amino acid.

Genetics deals with study of how genetic characters are passed from generations and they differ with time. New era in genetic studies combine with traditional genetics to understand the molecular genetics, that includes DNA analysis and examine the macromolecules in an organism. Various molecular techniques are used for isolation and examination of DNA or RNA that is transcribed from a specific gene. The molecular techniques used in molecular genetics are vast and few important areas are covered in this article.

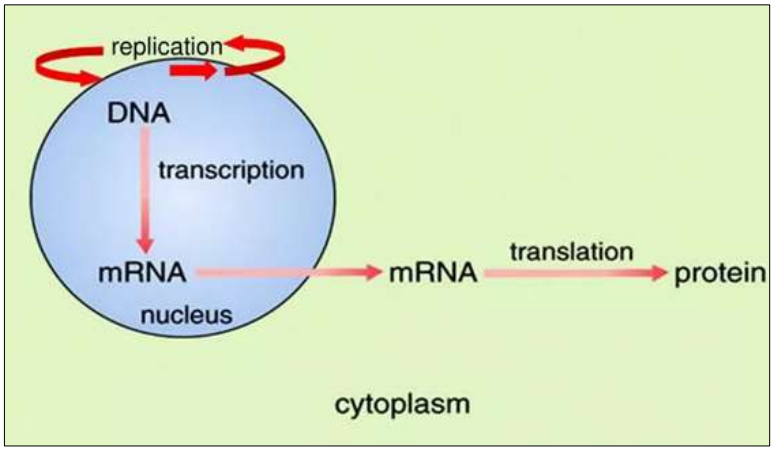

Fig 1: Central dogma of life- Replication, Transcription and Translation

1. Isolating Genomic DNA

Deoxyribonucleic acid (DNA) known as genetic material in all organisms. The isolation of DNA for the first time was carried out by Friedrich Miescher in 1869 [2]. The mechanism of extracting DNA from the cells of an organism is called DNA extraction. Blood, saliva or tissue are the biological samples used for the process of DNA extraction.

The process of DNA isolation involves certain steps like degradation of cells, evacuating the protein and other leftover contaminants and DNA purification, which allows free of other cellular components. Followed by use of purified DNA for other applications such as Polymerase Chain Reaction [3], cloning or sequencing.

The commonly used methods of DNA extraction are chemical, enzymatic lysis and mechanical. Precipitation, purification, and concentration are the commonly used steps in isolation of DNA. The most widely used methods practiced are alcohol precipitation, phenol-chloroform extraction, or silica-based purification [4]. In present day many commercially available DNA extraction kits are available based on the need of the genomic DNA kits are manufactured. Steps involved in DNA isolation- Cell lysis, Precipitation, Purification and Concentration (fig.2)

1. **Cell lysis:** The process of break open of the cells and DNA release is called cell lysis. For example, in bacterial cells, cell membrane disruption and DNA release is commonly done by utilising a solution salt such as SDS and a detergent. Enzymatic and mechanical method of DNA extraction is commonly used in animal and plant cells.
2. **DNA Precipitation:** After release of DNA from cell, remove other cell components like cell proteins and contaminants. Commonly in this step precipitating agent like alcohol (such as ethanol or isopropanol), or a salt (such as ammonium acetate) are used. After this step we could find DNA pellet at the bottom of the solution and contaminants remain in the liquid.
3. **Purification of DNA:** After DNA precipitation, further purified of genetic material is done by using column-based methods. Example of such type are, silica-based spin columns which binds the DNA, washing of cell contaminants are done using centrifugation and helps in purification of DNA by spinning it down to the bottom of a tube.
4. **DNA Concentration:** Remove excess liquid present in the tube and the DNA becomes concentrated. Vaccum centrifugation or freeze drying is most common employed.

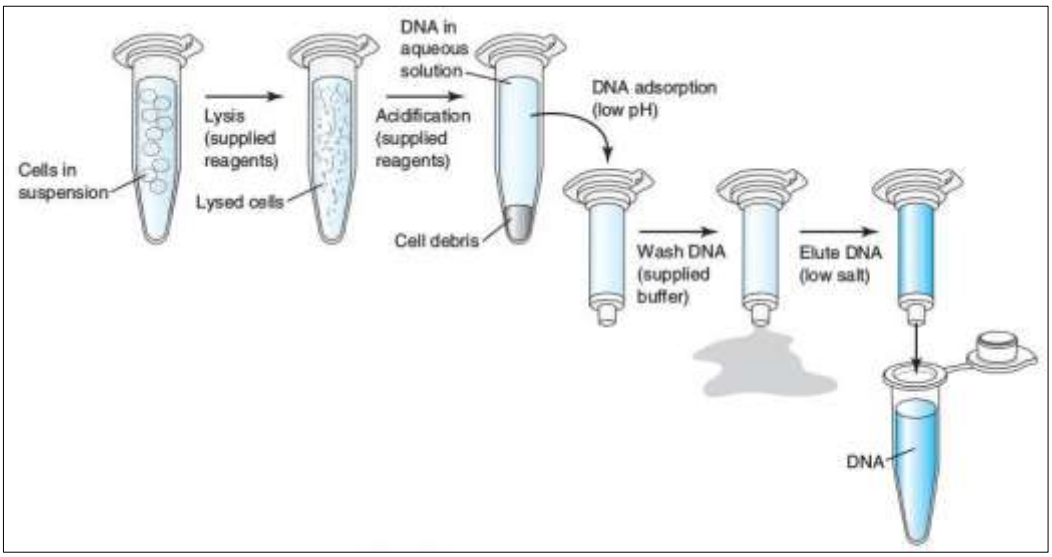

Fig 2: Steps involved in DNA isolation

The necessity for effective DNA extraction techniques has been underlined by the demand for molecular diagnostic assays in medical microbiology. In addition, automation of these methods is preferred. Several DNA isolation kits are available in the market for easy and quick isolation of DNA from variety of samples. Typical DNA isolation kit commonly follows alkaline lysis followed by using DNA binding matrix for incubation of DNA then DNA washing, and finally elution. Few commercially available DNA isolation kits are Thermofisher, Sigma-aldrich, Qiagen, Bio-Rad etc.

2. Polymerase Chain Reaction (PCR)

Molecular technique which synthesis large quantity of DNA target region in exponential manner invitro using enzymes for its synthesis is called Polymerase chain reaction. Synthesis of DNA using PCR is very much similar to *in vivo* DNA synthesis (in the cells) and it utilises DNA polymerase enzyme for its synthesis [5].

Kary Mullis first used PCR in the mid 80's [6]. Among the advanced molecular technique PCR helps in the characterization and identification of various organisms like bacteria, virus, fungus and parasites.

Principle of PCR

PCR is a powerful and widely utilized technique that has improved our knowledge about various genes. In cells we could find many thousands of genes. This makes individual gene extraction and analysis difficult. Therefore, this molecular technique is a boon for human kins as it enables the copying of specific DNA sequences, often corresponding to genes or regions of genes, from genomic DNA. For this we need to have a knowledge of a portion of the DNA sequence at each end of the region that needs to be replicated. The desired sequence of DNA is amplified more than one million times by PCR and takes predominant step in the process. Therefore, we can obtain sufficient amount of DNA for detailed analysis or manipulation of the replicated gene [7].

Components of PCR reaction

Template DNA: Single or double stranded DNA can be used as DNA template.

Oligonucleotide Primers: Synthetic oligonucleotides in pair must single stranded, short, and complementary to opposite strands. Standard PCR reaction contains each primer quantity of 0.1 - 0.5µM, which is sufficient for amplification of a 1-kb segment of DNA in 30 cycles. Standard amount of primers need to be used in reaction. Higher concentrations of primer favour unwanted priming and may lead to nonspecific amplification.

Thermostable DNA polymerase: DNA synthesis using PCR id temperature dependent, so DNA polymerase should be thermostable to withstand the denaturation temperatures (94-95 ºC). For the reaction to occure Taq polymerase pf 0.5-0.25 units are used in a standard 25-50µl reaction.

Deoxynucleoside triphoshates (dNTPs): dNTPs like Deoxycytidine triphosphate (dCTP), Deoxythymidine triphosphate (dTTP), Deoxyadenosine triphosphate (dATP) and Deoxy-guanosine triphosphate (dGTP) (200-250 µM each) of equimolar concentration are used standard PCRs.

Divalent Cations: Activation of thermostable polymerases require free divalent cations, among those group Mg+2 is most commonly used. Mg+2 binds to dNTPs and oligonucleotides. Routinely 1.5 mM concentration of Mg+2 is used. If concentration of Mg+2 is in excess it leads to non-specific amplification products and we could see decrese in the yield if Mg2+ is insufficient [8].

Buffer: Standard PCR mixture will have pH of 8.3 - 8.8 at room temperature. To maintain the PH of the reaction mixture buffers are used [9].

Monovalent cations: In standard PCR mix for amplification of DNA segments >500 bp in length require 50 mM of KCl as monovalent cation [10].

According to many researchers, inclusion of 10% dimethyl sulfoxide (DMSO) chemical in the buffer containing Taq polymerase will increase the efficiency of reaction [11, 12].

Procedure

- Sample used for the reaction includes DNA isolated from Blood, tissue (Fresh or frozen unfixed), Cytology smears, body fluids and stained sections and saliva etc., [13]
- DNA thermal cycler is used for amplification of DNA.
- Components in reaction mixture: A target sequence of 100-500 base pair length, 50 mM KCl, 10 mM Tris.HCI (pH of 8.4 at room temp), 1.5 mM MgCl2, 100 µg/ml Gelatine, 0.25 µM of each Primer, 200 µM of each Deoxynucleotide Triphoshates (dATP, dCTP, dGTP, and dTTP) and 2.5’ units of Taq Polymerase [14].

Steps involved in amplification process [15]

1. Template denaturation at temperature of 94 ºC
2. Oligonucleotide primers are annealed to single stranded target sequences
3. Annealed primers undergo extension using thermostable DNA polymerase

Types of PCR

1. **Multiplex PCR:** The type of PCR where one pair of primers are used for PCR reaction. The aim of this type of PCR is to simultaneously amplified many DNA sequences (usually exonic sequences). This PCR technique is commonly used for micro-satellite and genetic screening.
2. **Nested PCR:** Nested PCR, which is a series of two PCRs, each with two primers flanking the same sequence. The first PCR amplifies a sequence as observed in any PCR experiment. Subsequently, the second set of primers used in the second PCR anneals within the product of the first PCR, generating a second PCR product of reduced length compared to the first one. The technique, due to its utilization of four specific primers instead of two, exhibits more specificity when compared to regular PCR.
3. **Multiplex RT-PCR:** It is a frequently used technique for determining tissue-specific gene expression patterns, as well as semi-quantitative characterization of gene expression levels. Multiplex RT-PCR is typically carried out to assess the alterations in gene expression levels across a series of tissue types, during different levels of cellular differentiation and while using experimental treatments specifically. By simultaneously amplifying multiple target genes in a single reaction, this technique allows for efficient and comparative analysis of gene expression changes under different conditions.
4. **Real time PCR:** The Quantitative PCR (qPCR) is another name for real-time PCR, relies on the detection and measurement of a fluorescent reporter signal. Fluorescent signal increases accordingly to the quantity of PCR product produced during the amplification reaction. As the PCR progresses, the fluorescent signal accumulates and the system continuously monitors the fluorescence in real-time. In this method initial amount of the target DNA or RNA template in the reaction is quantified, making real-time PCR a powerful tool for precise and quantitative analysis of gene expression, viral load and various other applications in molecular biology.
5. **PCR-RFLP:** Polymerase Chain Reaction- Restriction Fragment Length Polymorphism is a molecular method for differentiating organisms based on patterns produced when their DNA is cut by particular restriction enzymes. The length of the fragment formed when DNA is digested by a restriction enzyme depends on whether two organisms have differing distances between the sites of cleavage of a certain restriction endonuclease. The resulting patterns' similarity can be used to differentiate across species.

PCR application

1. Using genome-specific primer pairs to identify pathogens and microbes in clinical samples
2. Diagnosis of phenylketonuria, haemophilia, sickle cell disease, and thalassemia, among other hereditary illnesses.
3. The detection of point mutations, insertions, and deletions in genes.
4. Checking for undiscovered mutations in certain genes
5. Determination and evaluation of eukaryotic DNA mutations.
6. Polymorphisms and expression of gene etc., [16]

3. Restriction Digest and DNA Ligation

Restriction enzymes ate DNA cutting enzymes and are used in Restriction digestion. Each enzyme after identifying the target sequence cleave the double stranded DNA at or near those sequences. Additionally, they are a part of the defensive mechanisms used by bacteria against viruses and other sources of bacterial DNA. Numerous restriction enzymes produce ends with single-stranded DNA overhangs by making staggered cuts. Some, however, result in blunt ends.

DNA ligase is most extensively used DNA joining enzyme. Ligase helps in linking the broken molecule of DNA if the ends of two DNA strands are identical.

Differentiation in composition of subunits, co-factor requirements and properties of DNA-cleavage made the early division of REases (17, 18, 19, 20) into Type I restriction enzymes which show both activities like restriction and DNA modification (Escherichia coli EcoKI, EcoBI), Type II enzymes and the methyltransferases that modify them function as independent proteins (EcoRI, HindII).Subsequently, Type III enzymes are similar to Type I, possess both modification and restriction activities (EcoP15I, EcoP1I) and the Type IV modification-dependent REases (Mcr and Mrr) were also determined as various classes (19, 20, 21).

4. Cloning DNA

DNA cloning a molecular biology technique widely used for making many identical copies of a piece of target DNA. Plasmids are found in variety of bacteria and are extrachromosomal DNA components. They normally exist in vast numbers within a cell and are double-stranded, circular, small (a few thousand base pairs), independent replicators. In cloning the target gene is inserted into plasmid. DNA "cut and paste" enzymes are used for the insertion, which results in a molecule of recombinant DNA, or DNA that has been put together from fragments from various sources.

Various steps followed in cloning

- Split open the plasmid with the aid of restriction enzymes and DNA ligase paste the target gene.
- Bacterial insertion of Plasmid. Insert the plasmid into bacteria
- Production of large number of identical copies of target DNA fragment

5. Agarose Gel Electrophoresis

Electrophoresis is a molecular technique was first developed by Arne Tiselius in 1937. The most typical applications of gel electrophoresis are for the separation and purification of proteins and nucleic acids.

In this technique charged molecules in an electric field can be separated from one another and are analysed. The size, shape, charge, and temperature of charged particles during electrophoretic separation all affect their mobility. Two electrodes, the anode and cathode, are linked by an electrolyte in the electrophoretic system. Agarose or polyacrylamide are used to make the gel. The seaweed-derived agar is useful for isolating DNA fragments with sizes ranging from a few hundred base pairs to roughly 20 kilobases. When it comes to proteins and tiny DNA fragments, polyacrylamide is preferable.

A intricate web of polymeric molecules forms an agarose gel. The type and amount of agarose used, as well as the makeup of the buffer, all affect the average pore size. Smaller molecules can be isolated from larger ones if the gel's pore size is tiny.

After polymerase chain reaction (PCR)-assisted DNA amplification, agarose gel electrophoresis is frequently used to separate limited genomic DNA or RNA in preparation for Southern and Northern analyses, respectively.

Equipments required for AGE

Gel casting trays, Sample comb, Electrophoresis buffer, Loading buffer, Ethidium bromide (EtBr), and Transilluminator are some of the electrophoresis equipment used in AGE.

Agarose is melted in the presence of the handy buffer until a clear, transparent solution is obtained to create agarose gels. The melted gel is then put onto a tray and given time to set. After solidification, place the DNA samples in the sample wells and run the gel at the voltage and for the duration necessary to achieve the best possible separation. DNA moves away from the cathode and towards the anode when an electric field is applied across the gel. The separated bands on the agarose gel are tagged or coloured for interpretation once the gel has finished running. Exposing DNA to the dye EtBr is one way to stain it. When exposed to ultraviolet (UV) light, EtBr intercalates between the stacking bases of nucleic acids and fluoresces red-orange. Because EtBr causes cancer, it should be treated carefully. Voltage, the make-up of the electrophoresis buffer, agarose concentration, molecular size, and DNA confirmation all have an impact on how DNA migrates through agarose gel. [22].

6. Blotting and Hybridization

DNA, RNA and protein are the biological macromolecules. The investigative technique in which transfer of macromolecules from a gel to the solid surface of an immobilised membrane is done in order to detect and identify the biological macromolecules is known as Blotting.

Principle of Blotting

The blotting methods are simple and commonly consist of four separate steps:

The separation of protein or nucleic acid fragments by electrophoresis, immobilisation on paper support and transfer to, analytical probe's tethering to the target molecule on paper, image of the bound probe.

Different types of Blotting

Southern Blotting: This technique identifies DNA fragments by using DNA probes that form hydrogen bonds with complementary chromosomal DNA fragments [23].

Northern blotting: Enables the identification and measurement of individual messenger RNA (mRNA) molecules following their hybridization to the DNA sequences from which they were derived. [24].

Western blotting: Allows specific antibodies used as analytical probes to identify certain proteins [25].

DNA hybridization is a molecular genetic process in which DNA single strands from two distinct species are allowed to combine to generate hybrid double helices. By comparing the base pair sequences of two species, one might infer how closely related they are to one another. By looking at how similar or different the DNA base pair sequences are, these hybrid DNA segments can be used to assess how closely

related different animals are to one another in terms of evolution.

Heating DNA strands from two distinct species to 86° C [186.8° F] starts the hybridization process. The hydrogen bonds between all complementary base pairs are subsequently broken as a result. Numerous segments of single-stranded DNA are produced as a result. Next, combine the single-stranded DNA from the two species, and let them slowly cool. DNA strands from both species that are similar to one another will start to chemically bind or re-anneal at base pairs that are complimentary by re-forming hydrogen bonds.

The resulting hybrid DNA is then heated, and it is noted at what temperature the DNA reverts to single strandedness. This method makes use of DNA's capacity to absorb UV radiation. DNA's single strands absorb UV radiation more effectively than its double strands. As a result, as more single strands are released, more UV light is absorbed, serving as evidence that the DNA strands have been separated.

Conclusions

In the modern molecular genetics period central dogma of life is the basic and play a key role in genetic information transfer from DNA to protein via transcription and translation. For the better understanding and diagnosis of genetic disorders and molecular basis of life one needs to go with application of molecular techniques.

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