MOLECULAR BIOTECHNOLOGICAL METHODS

1-16S and ITS rRNA Sequencing

16S rRNA is the component of the 30S small subunit of a prokaryotic ribosome. The prokaryotic 16S rRNA gene is approximately 1500 bp long, with nine variable regions interspersed between conserved regions. Variable regions of the 16S rRNA gene are frequently used for phylogenetic classification of genus or species in diverse microbial populations. The Internal Transcribed Spacer (ITS) region of the rRNA cistron is a commonly used DNA marker for identifying fungal species in metagenomic samples.

16S and ITS ribosomal RNA (rRNA) sequencing are common amplicon sequencing methods used to identify and compare bacteria or fungi. These techniques are identify and compare microorganisms from complex microbiomes or environments and are effective techniques to identify strains compared to traditional methods.

The advantages of using ribosomal RNA in molecular techniques are as follows:

-Ribosomes and ribosomal RNA are present in all cells.
-RNA genes are highly conserved in nature.
-Culturing of microbial cells is absent in the sequencing techniques.

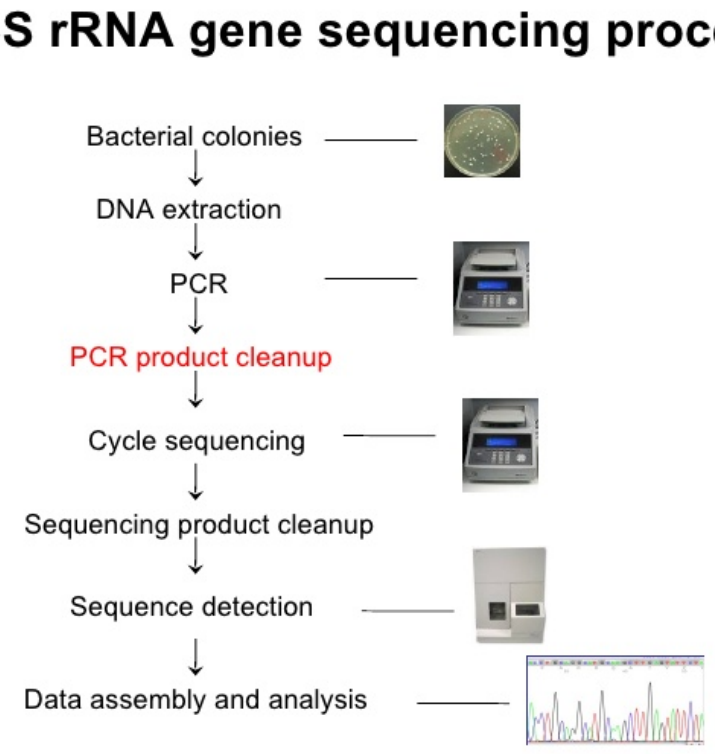


Figure1: Steps in rRNA sequencing

Steps in Ribosomal RNA Sequencing:

-Extraction of DNA

The genetic material of all living organisms contains information that is crucial for heredity. The DNA segments that carry this genetic information are called genes which are necessary for genetic analysis, which is used for scientific, medical, or forensic purposes. DNA is not free inside the nucleus of a cell. It is usually associated with different proteins and encased in a cellular membrane. Presence of these proteins, lipids, polysaccharides and some other organic or inorganic compounds in the DNA preparation can interfere with DNA analysis methods. Factors affecting the methods of DNA isolation are the age, source, and size of the sample. The presence of proteins, lipids, polysaccharides etc. during DNA preparation can interfere with DNA analysis methods by reducing the quality of DNA. The extraction methods to efficiently purify DNA from various sources have to be adapted depending on factors such as sample size, the freshness of the sample, and the biochemical content of the cells from which DNA is being extracted. The isolation method must vary depending on the size of sample. In the case of bacteria, the main biochemicals present in a cell extract are protein, DNA and RNA.

-Polymerase Chain Reaction

PCR is a rapid, automated technique used for the amplification of specific DNA sequences, It has gained over nucleic acid based detection techniques due to its simplicity, specificity, rapidity and sensitivity. In this technique only the DNA of the organism is examined, not the entire viable microorganism.

-Agarose Gel Electrophoresis

Electrophoresis is a technique used in the laboratory for separating charged molecules. DNA is negatively charged and it can be moved through an agarose matrix by means of electric current. Shorter molecules migrate more easily and move faster than longer molecules through the pores of the gel and this process is called sieving. The gel might be used to look at the DNA in order to quantify it or to isolate a particular band. The DNA can be visualized in the gel by the addition of ethidium bromide. It is an intercalating agent which intercalates between nucleic acid bases and allows the convenient detection of DNA fragments in gel. When exposed to UV light, it will fluoresce with an orange color. After the running of DNA through an EtBr-treated gel, any band containing more than ~20 ng DNA becomes distinctly visible under UV light. The migration rate of the linear DNA fragments through agarose gel is proportional to the voltage applied to the system. As voltage increases, the speed of DNA also increases.

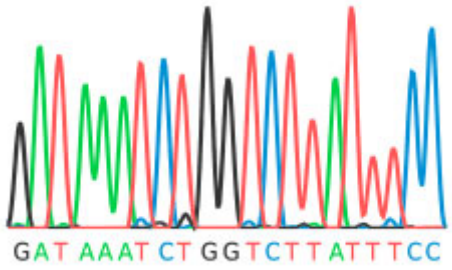
-Elution of DNA

Elution describes the extraction of specific bands of DNA from agarose gels in which they are separated through electrophoresis. The first step in extracting DNA is identifying the DNA band which is to extract, by illuminating under UV light. The extracted DNA precipitates out from the solution. Low melting point agarose is widely employed for the separation of DNA from agarose. Low melting point agarose melts at a lower temperature than standard agarose since it does not denature DNA structure.

-Sequencing and Identification

PCR products are purified and sequenced. **DNA sequencing** is the process of determining the nucleic acid sequence – the order of nucleotides in DNA. It includes any method or technology that is used to determine the order of the four bases: adenine, guanine, cytosine, and thymine. Obtained sequences are analysed and identified using DNA bioinformatics platforms (BLAST).

BLAST (Basic Local Alignment Search Tool) is the program compares nucleotide or protein sequences to sequence databases and calculates the statistical significance of matches. BLAST can be used to infer functional and evolutionary relationships between sequences as well as help identify members of gene families.



View of DNA Sequencing

2-Denaturing Gradient Gel Electrophoresis (DGGE)

Denaturing Gradient Gel Electrophoresis (DGGE) is a technique used to separate short- to medium-length DNA fragments based on their melting characteristics. In this method, partial 16S rDNA-amplified fragments of identical length but different sequence can be resolved electrophoretically because of their different melting behavior in a gel system containing a gradient of denaturants. DGGE were developed to analyze microbial communities in fermented milk products based on sequence-specific distinctions of 16S rRNA amplicons produced by PCR. If the total DNA of a microbial community is used in PCR amplification, these techniques can provide the profile of the genetic diversity of the dominant populations. If total RNA is used instead, the profiles reveal the metabolically active populations. Both PCR–DGGE and PCR–TGGE are used to study the diversity and dynamics of microorganisms in food fermentations and to profile pathogens directly in food samples.

Principles of DGGE

- ✓ Polyacrylamide gel with denaturing urea and formamide gradient,
- ✓ DNA fragments of the same size but with different sequences,
- ✓ All of genes are obtained from different DNA samples.

- ✓ Denatured Gradient polyacrylamide is separated from each other by the application of electric field on the gel.
- ✓ The dominant species is determined from the bands displayed on the gel.
- ✓ The large number of bands indicates the diversity in the sample.
- ✓ Sequence analysis can also be performed by cutting the obtained bands from the gel.
- ✓ With the sequence analysis following the method and phylogenetic analysis applied to the bands, sufficient information about the microorganism community can be obtained.
- ✓ The DGGE method provides convenience in cases where there are large amounts of samples that need to be analyzed and for detecting time-dependent changes in the number of microorganisms

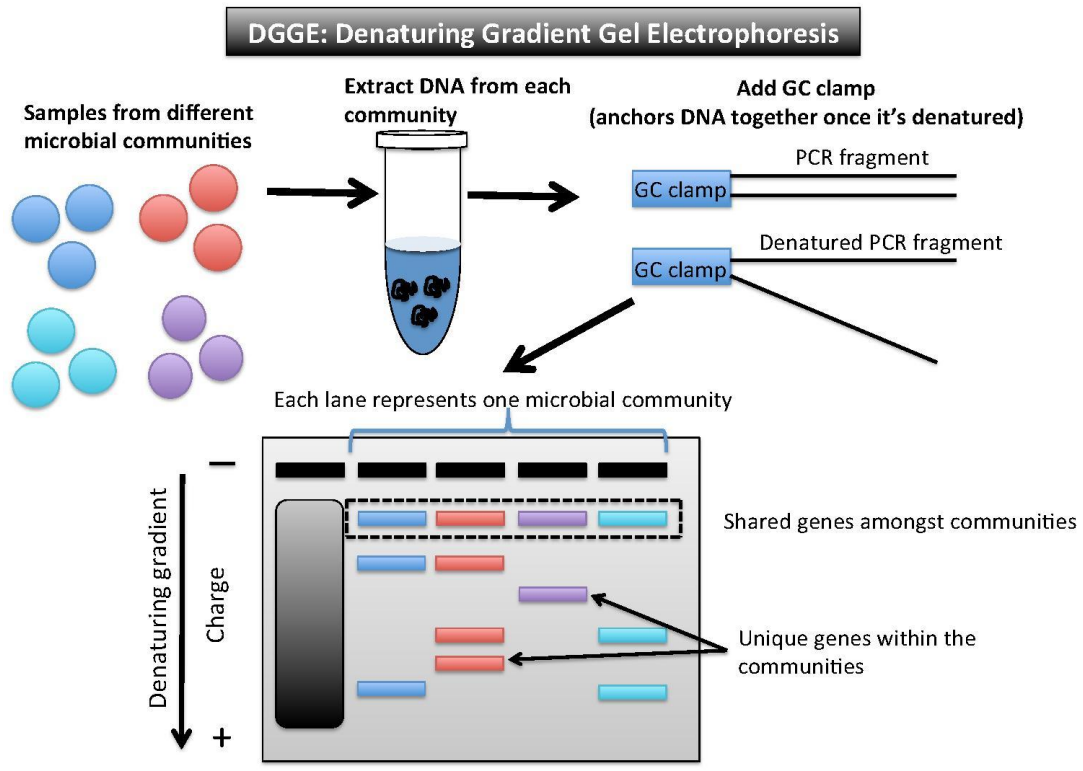


Figure. Process of DGGE

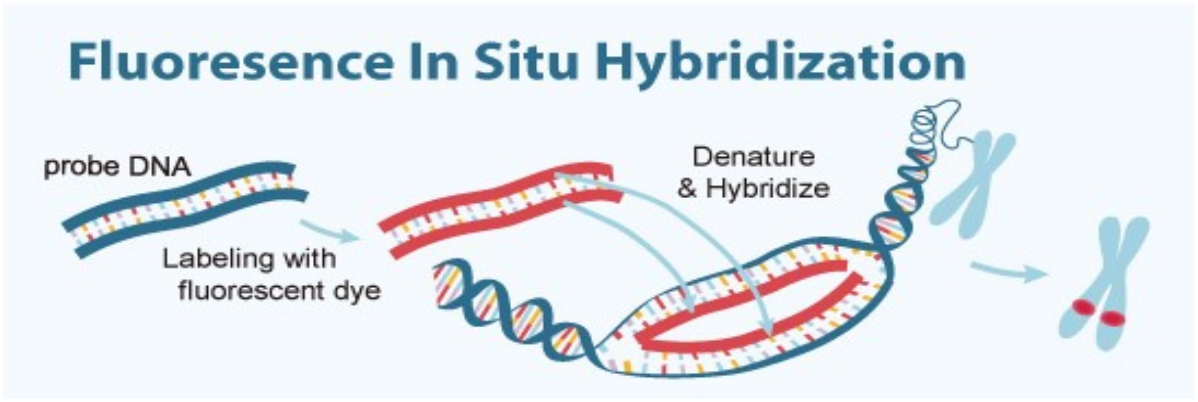
3-Fluorescent in Situ Hybridization (FISH)

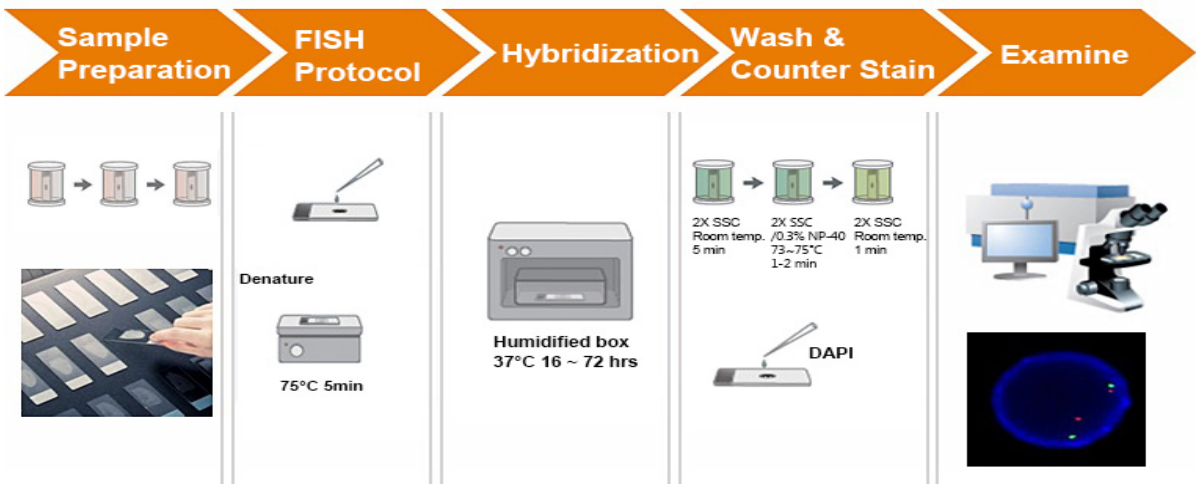
Fluorescence *in situ* hybridization (FISH) is a molecular cytogenetic technique that uses fluorescent probes that bind to only those parts of a nucleic acid sequence with a high degree of sequence complementarity.

Principles of FISH

It is the process of binding a fluorescent chemical to a labeled molecule that can recognize the target nucleic acid sequence.

- ❖ The labeled molecule is called a probe.
- ❖ The process of preparing the probe to allow it to bind to the chromosomes in the target DNA sequence on the chromosome is called hybridization.
- ❖ Since the target DNA probe attached to the chromosome is fluorescent, the gene is present if it irradiates under UV light; if it does not radiate, it is concluded that the gene particle is lost. Each probe labeled with a fluorescent molecule is specific to the targeted region of the chromosome.
- ✓ The method for detecting the origin of the target mRNA can be described as in situ hybridization. It is a precise and easy method.
- ✓ The location of the probe is determined by hybridization of the desired mRNA to the labeled nucleic acid probe
- ✓ DNA, RNA or oligonucleotides can be used as probes. DNA probes show high specific activity but must be denatured before use. RNA probes show low activity and can be destroyed by RNAases. Oligonucleotide probes are created complementary to the target sequence.





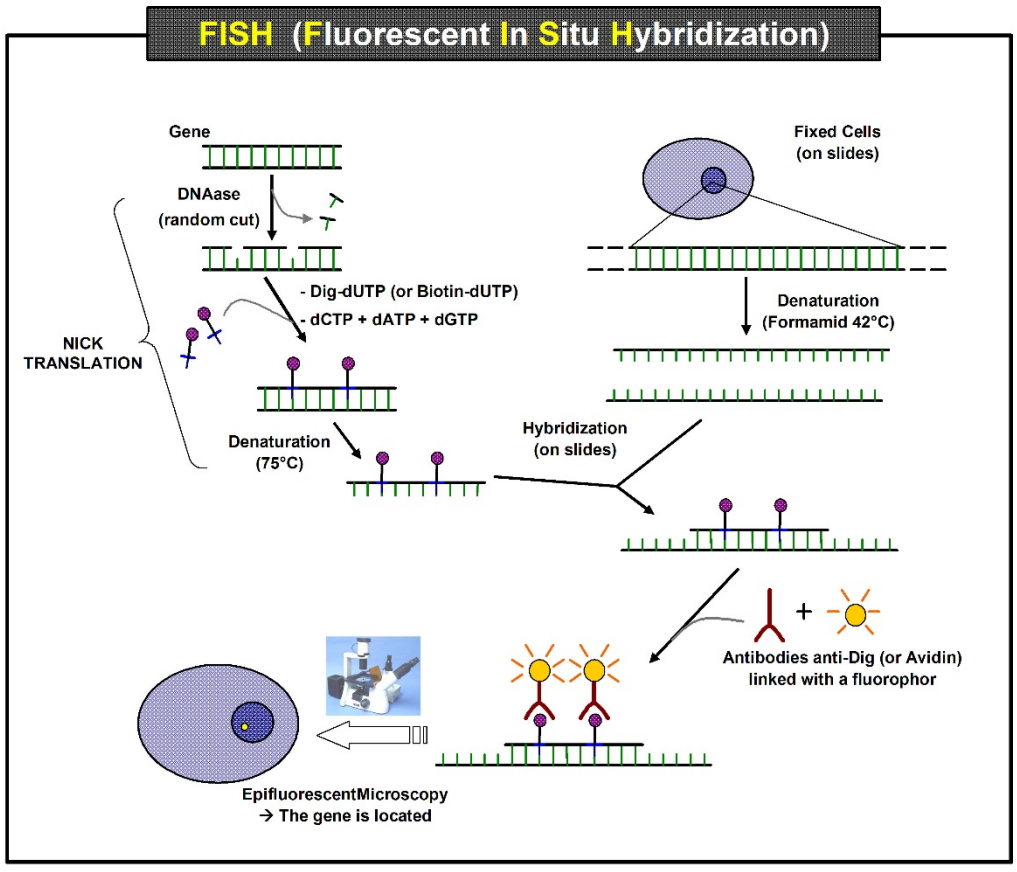


Figure. Process of FISH

4- 2D Gel Electrophoresis

Two-dimensional (2D) gel electrophoresis (abbreviated as **2-DE** or **2-D electrophoresis)**, is a form of gel electrophoresis commonly used to analyze proteins. Mixtures of proteins are separated by two properties in two dimensions on 2D gels.

2-D electrophoresis begins with electrophoresis in the first dimension and then separates the molecules perpendicularly from the first to create an electropherogram in the second dimension. In electrophoresis in the first dimension, molecules are separated linearly according to their isoelectric point. In the second dimension, the molecules are then separated at 90 degrees from the first electropherogram according to molecular mass

2-D gel electrophoresis is the primary technique for proteomics work. It separates the complex mixture of samples using two different properties of the proteins. In the first dimension, proteins are separated by the pI value and in the second dimension by the relative molecular weight.

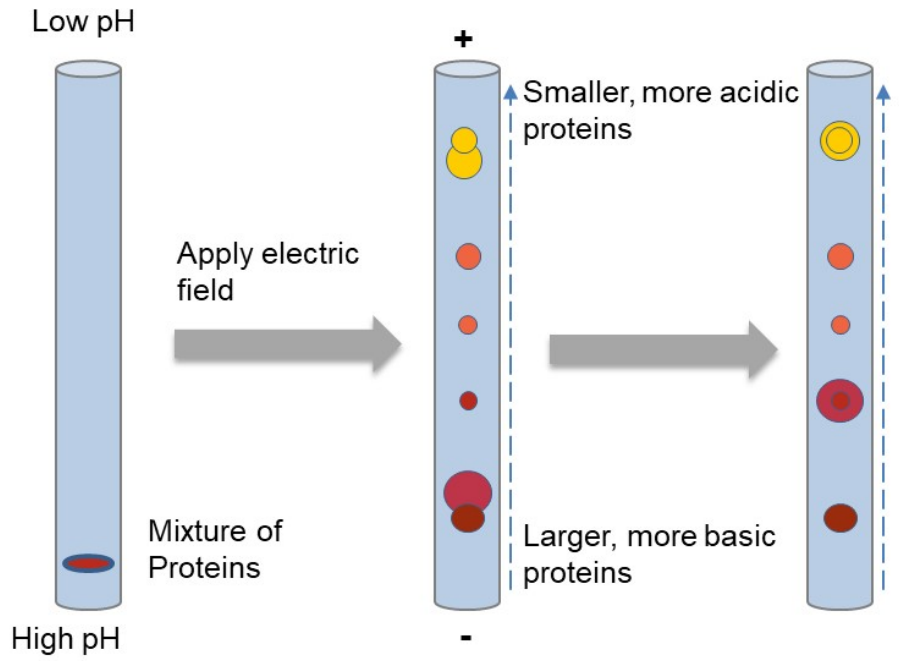


Figure. Principle of 2D gel electrophoresis