BRIEF CONTENT OF LECTURES ON DISCIPLINE “MODERN METHODS OF BIOTECHNOLOGY

Specialty McS Biotechnology

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Lecture. 1. Introduction to modern methods. The subject of modern BT methods. The use of methods in different biotechnology areas (agricultural biotechnology and breeding, microbial and environmental biotechnology).

The subject of modern BT methods

The use of methods in different BT areas.

Methods of study of membrane structures

Development of methods for cell disruption

BT includes

1. New methods of studies of living organisms
2. new application of results obtained by using these methods,

new philosophical and methodological approaches to study the living organisms (often said that biotechnology has created a new "ideology ").

3. Modern BT is interdisciplinary science . Methods used in BT directed to study:
4. the physical,
5. chemical,
6. biophysical,
7. biochemical properties and principles of the functioning of living systems.
8. Among them, there are methods that are used for a long time (centrifugation , spectrophotometry , various types of chromatography , immunological methods , etc.).
9. However, these methods still remain effective in the study of biological objectives.

Innovative BT high-tech methods:

using of them allows to obtain the results dramatically accelerated progress in biology and BT.

- The most significant of these modern methods

genetic engineering is changing the target of genes of an organism by manipulating its DNA. Methods of transferring genetic material from one object to another.

At the present time In experiment any combination of genetic elements obtained from different biological systems can be received.

3. Engineering enzymology

Is a change of the enzymes properties with a view to their use in the food, pharmaceutical and chemical industries.

For example, with the use of enzyme glycoseizomerase converting glucose into fructose assumed to get commercially the end product (diabetics).

3. One of the most important approaches - immobilization of enzymes or them containing cells - fastening on cellulose, collagen gel-forming materials, or other media. In the immobilized state enzyme function is improved or modified and increased their stability.

4. Methods of sequencing the nucleotide sequences of the NA.

This allows to study the primary structure of eukaryotic genomes with a size of tens of billions of base pairs.

5. Methods of analysis of the functional role of genes and other DNA sites.

This allows to establish differential activity of the genome during ontogeny

6. Cultivation of plant or animal cells on nutrient media. It is necessary for mass production of products (for example , medicinal ginseng glycosides cultivator with cells of the plant). From cultured plant cells, whole plants can be obtained which completely identical in hereditary traits (clones) .

7. industrial production of biological products on a large scale (e.g. , bacterial preparations for animal feed), or vice versa , in miniscule saturating the world market amounts (expensive drugs produced in gram amounts or even milligram),

the development of industrial biotechnology apparatus (fermentors , bioreactors) and production processes

8. Isolation, purification , chemical modification and stabilization of biotechnology products using modern methods (ion exchange , affinity and gel chromatography , electrophoresis, isoelectric focusing, isotachophoresis , immunochemical methods, etc.)

9. Biotechnology ecosystem (eco-engineering)

10 . The enormous potential of computer-based methods of studying NC , proteins and regulatory systems of cells and organisms

So, The rapid progress in various fields of science , related to biology , will lead to an improvement in the appearance of old and new techniques that will significantly accelerate the development of BT .

In this regard, we must know the basic methods of BT , which determine the development of the scientific and practical activities of man.

BT methods used in the following areas:

Agriculture .
BT pesticides, replacing pesticides, for example, the use of natural enemies of insects - pests or weeds;
Development of resistant pathogens , or virus-free plants;
The creation of new breeds of genetically engineered
Microbial biomass and other feed additives for animals;
New methods of prevention (genetically engineered vaccines) and treatment of diseases of farm animals.
2 . **Medicine**
provides new antibiotics, vaccines, therapeutic serum -based monoclonal antibodies, hormones, blood factors synthesized in microbial cultures using genetic engineering techniques and others.
3. **Food processing industry**
sweeteners , aromatics and flavorings Supplements (добавки), is not obtained by chemical synthesis, using a microorganism or cultured plant cells ;
• food enzymes ;
• Regulators - substances prolonging the shelf life of products.
4. Energetics - production of renewable fuels.
5 . **Mining (BIOGEOTECHNOLOGY)** leaching of metals from ores by microorganisms , microbial separation of water-oil emulsions;
6 . **The recovery of residual oil** from the wells by injecting therein viscous solutions microbial biopolymers.
5. Mining industry (biogeotechnology): leaching of metals from ores with the help of microorganisms; microbial separation of water-oil emulsions**;**
6. Recovery of residual oil from wells by pumping viscous solutions of microbial biopolymers into them.

LECTURE 2. Methods of studying of membrane structures. 2.1. Development of methods for cell disruption. Methods of identification of subcellular fractions.

1. Methods of Studying Membrane Structures
a) Microscopy-Based Methods
1. Optical microscopy (fluorescence, confocal, super-resolution)
Visualize membranes with fluorescent dyes/probes.
Super-resolution (STED, PALM, STORM) → nanometer-scale imaging.
2. Electron microscopy (TEM, SEM, Cryo-EM)
TEM: Internal ultrastructure of membranes.
SEM: Surface topology of cells and membranes.
Cryo-EM: Native state, high-resolution membrane protein complexes.
3. Atomic Force Microscopy (AFM)
Nanometer-scale surface imaging.
Measures membrane *stiffness, roughness, mechanical properties.*
b) Biophysical Methods
- **X-ray diffraction & Neutron scattering**
  - Reveal membrane bilayer organization, thickness, lipid phases.
- **NMR spectroscopy**
  - Structure and dynamics of membrane lipids and proteins.
- **Surface Plasmon Resonance (SPR)**
  - Real-time study of membrane-protein interactions.
- **Fluorescence techniques (FRAP, FRET)**
  - FRAP: Membrane fluidity (lateral diffusion of lipids/proteins).
  - FRET: Protein–protein or lipid–protein interactions.

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2. Development of Methods for Cell Disruption
To study membranes and organelles, cells must be broken open while preserving functional biomolecules.
Physical Methods
Mechanical homogenization (Potter–Elvehjem, French press) → shear force breaks cells.
Sonication → ultrasonic vibrations disrupt membranes.
Freeze–thaw cycles → ice crystals rupture cells.
Osmotic shock → sudden change in osmotic pressure disrupts fragile cells.
Chemical Methods
Detergents (Triton X-100, SDS, CHAPS) solubilize membranes**.**
Organic solvents destabilize lipid bilayers.
Enzymatic Methods
Lysozyme, cellulase, chitinase degrade cell walls.

Often combined with mild physical treatment for prokaryotic cells.

3. Methods of Identification of Subcellular Fractions
After disruption, separation and identification of cellular components is key.
a) Centrifugation-based
Differential centrifugation
Sequential spins → nuclei, mitochondria, lysosomes, microsomes, cytosol.
Density-gradient centrifugation (sucrose/cesium chloride gradients)
Higher resolution separation of organelles and membrane vesicles.
b) Electrophoresis-Based
SDS-PAGE: Identifies proteins from subcellular fractions.
2D gel electrophoresis: Separates proteins by pI and molecular weight.
c) Immunological methods
Western blotting with organelle-specific antibodies.
Immunofluorescence: Uses antibodies to confirm localization.
d) Marker enzyme analysis
Specific enzymes indicate purity of fractions:
Cytochrome c oxidase → mitochondria.
Acid phosphatase → lysosomes.
Catalase → peroxisomes.
Glucose-6-phosphatase → ER microsomes.
e) Advanced Methods
Proteomics (LC-MS/MS) → protein profiles of organelles.
Lipidomics → membrane lipid composition.
Flow cytometry & cell sorting (FACS) → isolate organelles/vesicles.

Summary:
Membrane structures → studied by microscopy (EM, AFM, super-resolution) & biophysical techniques (XRD, NMR, FRAP/FRET).
Cell disruption → physical, chemical, enzymatic methods depending on sample.
Subcellular fractions → identified by centrifugation, immunological markers, enzyme assays, and omics approaches.

LECTURE 3 Biophysical methods for studying membrane structures. The method of electron paramagnetic resonance (EPR) spectroscopy. Nuclear magnetic resonance spectroscopy (NMR spectroscopy) method. Surface plasmon resonance (SPR).

Biophysical methods
Electrophysiology
Hydrodynamics
Microscopy & Imaging
Atomic Force Microscopy (AFM).
Biophysical methods allow to study: the characterization of molecular structure, the measurement of molecular properties, and the observation of molecular behavior
A wide range of biophysical techniques are developed to study molecules in **crystals, in solution, in cells, and in organisms**.
These biophysical techniques provide information about: ***the electronic structure, size, shape, dynamics, polarity, and modes of interaction of biological molecules***.
Some of the most exciting **Biophysical Techniques** provide
images of cells,
subcellular structures, and
even individual molecule
It is now possible, for example,
to directly observe the biological behavior and physical properties of single protein or DNA molecules within a living cell and
determine how the behavior of the single molecule influences the biological function of the organism.
Electrophysiology
Detection of Secretion by Electrochemical Methods
The basic requirements for the types of electrochemical experiments that are used at single
cells are two electrodes placed in a common solution, a voltage source, and a **picoammeter.** One of the electrodes, the reference electrode, maintains a constant potential.

Early in the development of electrochemical techniques, the normal hydrogen electrode (NHE) was accepted as a standard.

Electrophysiology

In practice, the NHE is difficult to use and, more commonly, the Ag/AgCl electrode is used. By convention, the NHE has a standard potential, or E^o, of 0 V. and other types of reference electrode's potentials are commonly compared to the

NHE. For example, the Ag/AgCl electrode has an E^o of 0.197 V versus the NHE, or $E^o = 0.197$ V

vs. NHE, in electrochemical terminology. The Ag/AgCl electrode consists of metallic Ag coated with AgCl in contact with an aqueous solution containing a fixed concentration of chloride ion.

This electrode configuration generates a stable potential and is insignificantly perturbed when small currents flow through it.

Hydrodynamics is complex and directly related to molecular size, shape, and flexibility of large biomolecules;

The behavior of large biomolecules such as :

proteins, carbohydrates, and nucleic acids in solution.

The most accessible developments in biophysics involved improvements in our ability to generate **images of cellular and molecular structures with dimensions from microns to nanometers.**

It is now possible to "see" individual molecules or cellular structures using atomic force,

electron, or confocal fluorescence microscopy.

Confocal Fluorescence Microscopy

Confocal microscopy offers several advantages over conventional optical microscopy, including

1. controllable depth of field,
2. the elimination of image degrading out-of-focus information, and
3. the ability to collect serial optical sections from thick samples.

The key to the confocal approach is the use of spatial filtering to eliminate out-of-focus light or flare in samples that are thicker than the plane of focus.

There has been a tremendous explosion in the popularity of confocal microscopy in recent years, due in part to the relative ease with which extremely high-quality images can be obtained from samples prepared for conventional optical microscopy, and in its great number of applications in many areas of current research interest, including biotechnology.

Confocal Fluorescence Microscopy

Root of Arabidopsis thaliana with green fluorescent protein decorating cell membrane and red fluorescent protein marking nuclei.

Atomic Force microscopy (AFM)

Allows measuring force of intermolecular interaction and bonds between a bacterium and another surface.

AFM is very useful in biological sciences because it can be used on living cells that are immersed in water.

AFM is particularly useful when the cantilever (support) is modified with chemical groups (e.g. amine or carboxylic groups), small beads (e.g. glass or latex), or even a bacterium.

AFM operates on a very different principle than other forms of microscopy, such as optical microscopy or electron microscopy.

The key component of an AFM is a cantilever (*консольная*) that bends in response to forces that it experiences as it touches another surface.

Forces as small as a few picoNewtons can be detected and probed with AFM.

Atomic Force Microscopy (AFM)

Atomic-force microscopy (**AFM**) or **scanning-force microscopy** (**SFM**) is a type of scanning probe microscopy (SPM), with demonstrated resolution on the order of fractions of a nanometer, more than 1000 times better than the optical diffraction limit. The information is gathered by "feeling" or "touching" the surface with a mechanical probe. Piezoelectric elements that facilitate tiny but accurate and precise movements on (electronic) command enable very precise scanning.

Abilities of Atomic Force Microscopy AFM

has three major abilities:

1. force measurement,
2. imaging, and
3. manipulation.

In force measurement, AFMs can be used to measure the forces between the probe and the sample as a function of their mutual separation. This can be applied to perform ***force spectroscopy.***

For imaging, the reaction of the probe to the forces that the sample imposes on it can be used to form an image of the three-dimensional shape (topography) of a sample surface at a high resolution. This is achieved by raster scanning the position of the sample with respect to the tip and recording the height of the probe that corresponds to a constant probe-sample interaction (see section topographic imaging in AFM for more details). The surface topography is commonly displayed as a ***pseudocolor*** plot.

Abilities of Atomic Force Microscopy AFM

In manipulation, the forces between tip and sample can also be used to change the properties of the sample in a controlled way. Examples of this include atomic manipulation, scanning probe lithography and local stimulation of cells.
Simultaneous with the acquisition of topographical images, other properties of the sample can be measured locally and displayed as an image, often with similarly high resolution. Examples of such properties are mechanical properties like stiffness or adhesion strength and electrical properties such as conductivity or surface potential. In fact, the majority of SPM techniques are extensions of AFM that use this modality.

LECTURE 4 Membranes structure study by choice and use of detergents and mechanisms of their effect.

Membranes are composed of a lipid bilayer and associated proteins. Lipids are amphipathic molecules in that they contain a polar head group and hydrophobic tails. In aqueous solutions lipids will aggregate such that the hydrophobic tails interact with other hydrophobic tails and the polar head groups are exposed to water. The two possible configurations are: 1) a spherical micelles with the hydrophobic tails pointed inward, or 2) a bilayer with the hydrophobic tails sandwiched between the polar head groups. The shape of the lipid and its amphipathic nature cause them to spontaneously form bilayers in aqueous solutions and accounts for the stability of membranes.
Proteins interact with this layer bilayer in several different fashions. Transmembrane proteins pass through the bilayer. A hydrophobic domain, typically an α-helix composed of amino acids with hydrophobic side-chains, interacts with the hydrophobic tails of the lipids and anchors the protein to the bilayer. Some transmembrane proteins will have multiple membrane spanning domains. Other membrane proteins are anchored to the lipid bilayer via fatty acids or lipids that are covalently attached to the protein. Other membrane are attached to the membrane by non-covalent association with other membrane proteins.
The condenser lens, is an **electromagnet instead of a glass.**
These electrons are differentially impeded by the various structures within the sample.
In other words, some of the electrons are scattered or absorbed by the atoms of the specimen. The electrons which pass through the sample are focused with a series of magnetic objective lens onto either a photographic plate or a fluorescent screen.
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spanning domains.
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Many cellular processes occur on membranes or in membrane-bound subcellular
compartments.
Approximately half of a cell's total protein is associated with membranes or
found in membrane-bound compartments. The study of these membrane associated processes may require the isolation of membranes or membrane proteins. The choice of tissue and membrane fraction (i.e., organelles) will depend in part on the phenomenon being studied.
Some cell types are better sources for certain types of membranes. The cells need to be disrupted by procedures which preserved the activity of interest (see next slide).
Disruption of cells or subcellular compartments will usually result in the
membranes forming vesicles.
The different types of membranes will have different sizes and densities based upon their lipid and protein composition and can be prepared by differential centrifugation, density gradient centrifugation or a combination of the two.
choice of cells or tissue
• choice of membrane fraction
• homogenization conditions
• preparation of membranes
♦ differential centrifugation
♦ density gradient centrifugation
• solubilization of membranes
• isolation of proteins
The exact isolation technique will depend upon the source and type of membrane being isolated as well as the desired purity.

Interaction of detergents with membrane structures
Methods for detergent removal

Detergents interact with both membrane lipids and proteins.
At low detergent to lipid ratios,
detergent monomers incorporate into the lipid bilayer without disrupting the membrane.
***As the detergent:lipid ratio increases*,**
lipids, because of their similar properties as detergents, will form **mixed micelles with the detergent.**
Therefore, the detergent concentration needs to be above the **CMC.** Detergent molecules also interact with the hydrophobic portions of the proteins and in effect replace the lipids.
This will lead to the proteins being coated with detergents and
prevent protein-protein interactions that would normally result **in protein precipitation.**
Protein solubilization occurs at or near **the CMC** for most detergents.
There are no general rules for choosing among the various types of detergents.
It is not possible to predict which detergent will be most useful for any particular application.
Pilot experiments to determine the optimal detergent as well as the optimal conditions for maximal protein solubilization are carried out.
Possible detergent effects
• protein structure
• protein activity
• interference with assays
• separation techniques
DETERGENT REMOVAL
A detergent is solubilized the protein of interest.
It is necessary to remove the detergent. In general, detergents are difficult to remove.
Therefore, when choosing a detergent is need also to consider how ease a detergent can be removed.
methods for detergent removal

1. Dialysis
2. Chromatography
3. Replacement

The other electrode in biological applications is typically a **carbon fiber microelectrode** and it is referred to as the *working electrode*. By controlling the surface potential of the working electrode and simultaneously measuring the amount of current passing through it, information concerning the charge transfer processes that occur at the interface of the carbon and solution is obtained.
The analysis of hydrodynamic behavior of large biomolecules provides important information about the structure, dynamics, and interactions of biomacromolecules.

LECTURE 5. Theme Western blotting method (protein Immunoblotting. Describe enzyme activity analysis by electrophoresis

Western blotting (sometimes called protein immunoblotting) or Western blotting is a widely used technique in molecular biology and immunogenetics for the identification of target proteins in a protein sample from tissue homogenate or extract sample. Additionally, this technique is also applied to *visualize, distinguish,* and *quantify different proteins in a complex protein combination*.

The Western blotting technique uses three main elements to achieve its goal of separating a specific protein from a complex (**Figure**):

1. Separation by size.
2. Transfer of the protein to solid support.
3. Labeling of the target protein using a primary and secondary antibody for visualization.

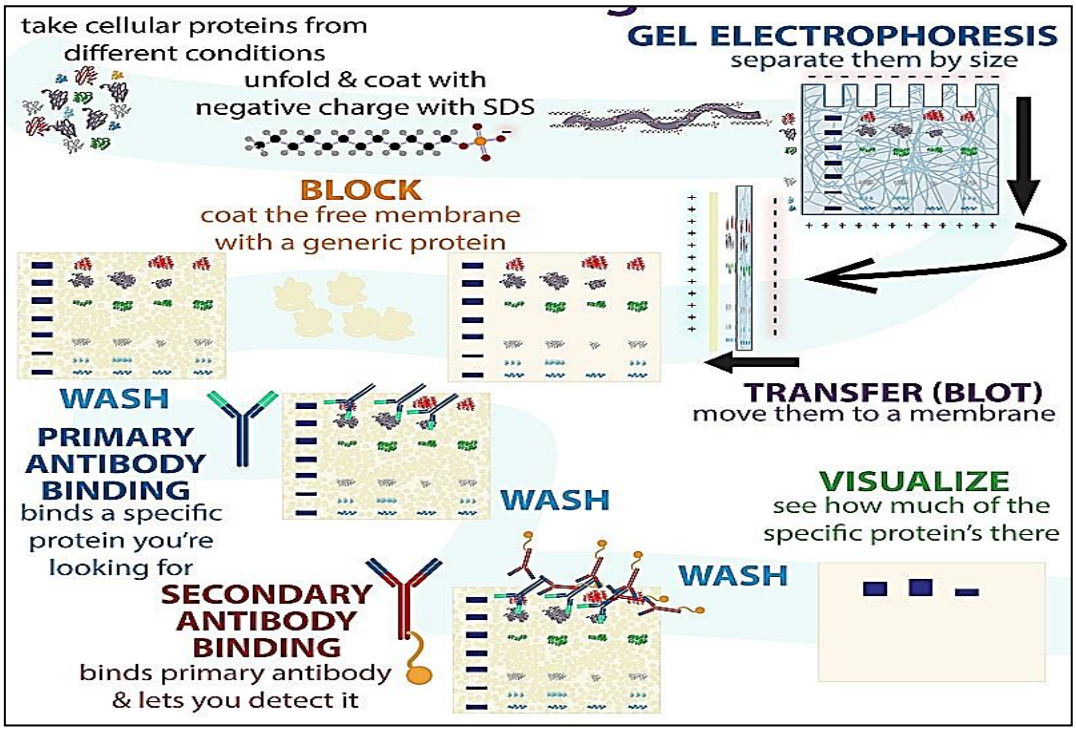

Figure. Western blotting workflow. From https://en.wikipedia.org/wiki/Western_blot#/media/File:Western_blot_workflow.jpg

A synthetic or animal antibody (known as the primary antibody) is created that recognizes and binds to a specific target protein. The electrophoresis membrane is washed in a solution containing the primary antibody before the excess antibody is washed away. A secondary antibody is added that recognizes and binds to the primary antibody. The secondary antibody is visualized using various techniques such as staining, immunofluorescence, and radioactivity, allowing for indirect detection of the specific target protein.

Other related methods include dot blot analysis, quantitative dot blot, immunohistochemistry, immunocytochemistry, where antibodies are used to detect proteins in tissues and cells using immunostaining and enzyme-linked immunosorbent assay (ELISA).

Procedure for Western blotting (**Figure**). The Western blotting method consists of gel electrophoresis to separate the proteins by their three-dimensional (3-D) structure or denatured proteins by the length of the polypeptide, followed by electrophoretic transfer to a membrane (usually PVDF or nitrocellulose) and an immunostaining procedure to visualize a specific protein on the blot membrane. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is commonly used for denaturing electrophoretic separation of proteins. Sodium dodecyl sulfate (SDS) is commonly applied as a buffer (as well as in the gel) to give all proteins present a uniform negative charge, since proteins can be positively, negatively, or neutrally charged. Protein samples are boiled prior to electrophoresis to denature the proteins present. This ensures separation of proteins by size and prevents degradation of the samples by proteases (enzymes that break down proteins). Following electrophoretic separation, the proteins are transferred to a membrane (usually nitrocellulose or PVDF). The membrane is then frequently stained with *Ponceau S* to
Total protein staining in Western blotting.
Blocking of proteins on membrane. Primary antibody Secondary antibody for binding a species-specific portion of the primary antibody. Radioactive detection of Western blotting.

Lecture 6 L. 6. Theme. Methods of Analysis and characterization of proteins

Proteins are purified by fractionation procedures, a series of independent steps in which the properties of protein of interest are utilized to separate it from other contaminating proteins.
General strategy of protein purification
Characteristic Procedure Solubility 1. Salting in 2. Salting out Ionic charge: 1. Ion exchange chromatography 2. Electrophoresis 3. Isoelectric focusing Polarity: 1. Adsorption chromatography 2. Paper chromatography 3. Hydrophobic interaction chromatography Molecular size: 1. Dialysis and ultrafiltration 2. Gel electrophoresis 3. Gel filtration chromatography 4. Ultracentrifugation Binding specificity: 1. Affinity chromatography .

- Assay of specific Proteins

In addition to measuring the total amount of protein, it is often necessary to estimate the **amount of a specific protein** in a mixture of proteins.

Measuring a specific protein will depend upon the availability of an assay that is specific for the protein of interest. Protein assays should be practical in addition to being specific and accurate.

- Specificity
- • Sensitivity
- • Accuracy
- (Quantification)
- • Rapid
- • Easy to Perform.

LECTURE 7 Main principles of electrophoresis

- GEL ELECTROPHORESIS
- SDS-PAGE
- Practical considerations.
- Electrophoresis,

like centrifugation, is a hydrodynamic technique.

A charged particle (i.e., molecule) in an electric field experiences a force that is proportional to the potential difference (E), or voltage, of the electric field and inversely proportional to the distance (d) between the electrodes.

(The potential difference divided by the distance (E/d) is referred to as the field strength.)

The force will also be proportional to the net charge of the molecule (q).

Therefore, the force experienced by the molecule can be expressed by the following equation:

The force of charged particle (i.e., molecule) experienced them in an electric field will by opposed by a frictional force (*сила трения*) (= fv),

where f is a frictional coefficient and v is the velocity of the particle

The frictional coefficient depends on the size (eg., r = radius) and shape of the molecule and the viscosity (η) of the medium.

For example, in the case of a sphere the frictional force is:

- A particle will move at a velocity (v) so that these two forces are equal, therefore:

or solving for v

This equation indicates that the mobility (i.e., velocity) of a molecule in an electric field

is proportional to the electric field (E/d), or more simply the applied voltage, and the net charge of the molecule.

The mobility of particle in an electric field is inversely proportional to a frictional coefficient (i.e., size and shape of the molecule and the viscosity of the medium), as indicated by the following equation:

mobility = (applied voltage)(net charge/(friction coefficient

Therefore, it is possible to derive information about the charge, size and shape of a molecule by its mobility in an electric field.

GEL ELECTROPHORESIS

Electrophoresis of macromolecules can be carried out in solution.

However, the ability to separate molecules is compromised by their diffusion.

Greater resolution is achieved if electrophoresis is carried out on semi-solid supports such as polyacrylamide or agarose gels.

Gels are formed by cross-linking polymers in aqueous medium.

This will form a 3-dimensional meshwork which the molecules must pass through.

Polyacrylamide is a common gel for protein electrophoresis whereas agarose is more commonly used for nucleic acids. *Agarose gels have a larger pore size than acrylamide gels and are better suited for larger macromolecules. However, either type of gel can be applied to either nucleic acids or proteins depending on the application.*

Gels are formed from long polymers in a cross-linked lattice.

The space between the polymers are the pores. Higher concentrations of the polymer will result in smaller average pore sizes. Polyacrylamide gels are formed by covalently cross-linking acrylamide monomers with bis-acrylamide with a free radical like persulfate ($SO4\cdot$). The cross-linking of the acrylamide polymers results in 'pores' of a defined size. The total acrylamide concentration and the ratio of bis-acrylamide to acrylamide will determine the average pore size. The polyacrylamide solution is poured into a mold and polymerized. This mold can be a cylindrical tube, but is usually a 'slab' poured between two glass plates

- EQUIPMENT. Equipment to conduct gel electrophoresis is relatively simple. They consist of a mold to form the gels, an apparatus to hold the gel and contain buffers, and a power supply capable of delivering the required voltage or current.

Discontinuous or "disc" electrophoresis.

The Laemmli discontinuous buffers are extensively used in gel electrophoresis. Discontinuous gels consist of two distinct gel regions referred
to as stacking gel (Штабелирующий) and separating gel and a Tris-glycine tank buffer.
The stacking gel has a lower acrylamide concentration, a lower pH and a lower ionic strength than the separating

Composition of Laemmli Gels

The lower ionic strength of the stacking gel results in a greater local electric field strength than in the separating gel.
The field strength difference combined with the lower acrylamide concentration results in proteins having a higher mobility in the stacking gel than in the separating gel.
In addition, the glycine in the tank buffer has a higher mobility in the separating gel than in the stacking gel because of the pH differences.
Therefore, proteins will migrate faster than the glycine in the stacking gel.
When proteins reach the separating gel their mobility is decreased because of the
increased acrylamide concentration and decreased field strength, whereas the increase in pH
results in glycine having a higher mobility.
All of these factors result in the proteins becoming compressed at the interface between the two gels and thus increasing resolution.
Resolution in non-discontinuous electrophoresis depends partially on the volume of the sample.
However, stacking also occurs at the interface of the sample and gel, especially if a high voltage is applied.

- SDS-PAGE

Polyacrylamide gel electrophoresis in the presence of SDS (sodium dodecyl sulfate) is the most common form of protein gel electrophoresis.
SDS completely disrupts protein-protein interactions and denatures almost all proteins resulting in a complete unfolding of proteins.
In addition, ß-mercaptoethanol (or other reducing agents) is often used to break disulfide bonds.
The SDS binds to the unfolded proteins giving all proteins a similar shape (i.e., random coil or extend conformation) and an uniform charge-to-mass ratio.
In other words, coating proteins with a negatively charged detergent minimizes the effects of a protein's net charge.
Therefore, during electrophoresis in the presence of SDS the mobility of a protein now depends primarily upon its size (i.e., mobility is inversely proportional to protein mass).

- SDS-PAGE

Mobility in SDS gel electrophoresis is expressed as a relative mobility (Rf).
The distance the protein migrated is compared to the length of the gel, or:
The length of the gel is often defined by the migration of a substance which is not impeded by the matrix such a small molecular weight tracking dye (eg., bromophenol blue).
This mobility can then be used to calculate the size of proteins.
Protein standards of known size are used to generate a standard curve by plotting the log of the molecular weight against the Rf values.

- Practical considerations.
- 1. Pour separating gel.
- 2. Pour stacking gel.
- 3. Load samples.
- 4. Apply electric field.
- 5. Stain or process gel.

Proteins to be analyzed by SDS-PAGE are solubilized in a sample buffer.
Typically contains 2% SDS and 5% β-mercaptoethanol and then boiled.
The reducing agent is omitted in situations where disulfide bonds need to be preserved.
When an enzyme activity will be measure following electrophoresis, a lower SDS concentration is used and the sample is not boiled.
The amount of protein that can be loaded onto a gel is limited. Overloading the gels results in the pores becoming plugged (*закупоривать*) and has an adverse effect on the electrophoresis.

- ISOELECTRIC FOCUSING

Isoelectric focusing (IEF) separates proteins based on their isoelectric points.
The isoelectric point is defined as the pH at which a protein has no net charge (i.e., the number of negative and positive charges are equal) and is a measure of the protein's net charge.
Separating proteins according to their net charge is accomplished by generating a pH gradient in an electric field.
The effect of protein size on mobility is minimized by carrying out the electrophoresis gels with large pore sizes such as low acrylamide concentrations (eg., 3.5%) or agarose.
This large pore size minimizes the molecular sieving.
A pH gradient is generated with carrier ampholytes.
These ampholytes are a mixture of aliphatic amines and either carboxylic or sulfonic
acid. They have a high buffering capacity, low molecular weight (300-600 Da) and a range of pKa values. Initially the pH of an ampholyte

solution will be the average of the pKa values of the mixture.
Application of an electric current causes the ampholytes to migrate toward the electrodes according to their charges. Ampholytes that have pKa values above the pH will be positively charged and those with pKa values below the pH will be negatively charged. As the ampholytes migrate this will result in changes in the local pH due to the buffering action of the ampholytes.
.
This change in the local pH will affect the charge on the ampholytes depending upon the pKa.
The ampholytes will continue to migrate until they reach a position in which the local pH equals their pKa (i.e., no net charge). The end result is a pH gradient in which the most basic ampholytes are found at the cathode, a dilute alkali solution (eg., NaOH), and the most acidic ampholytes are at the anode, a dilute acid solution (eg., H3PO4).
Carrier ampholytes with defined pH ranges can be purchased or prepared by isoelectric focusing.

- Proteins are also ampholytes and will migrate within the pH gradient until they reach a pH equal to their isoelectric point.
- The carrier ampholytes are needed since the protein concentration is generally not high enough to establish a stable pH gradient and the isoelectric points of the proteins may not be uniformly distributed along a pH gradient

IEF is an equilibrium phenomenon since the components of the system migrate until they have no net charge. As the system approaches equilibrium the resistance approaches infinity since there are no ions to conduct the current. However, the pH gradient will start to break down before true equilibrium is reached and the ampholytes will migrate into the anode and cathode buffers.
This gradient breakdown is accompanied by a lowering of the resistance.
Therefore, the progress of IEF can be followed by performing the electrophoresis under
constant voltage and monitoring the current. Initially the current will rapidly drop in concordance with the rapid migration of the ampholytes.
As the ampholytes lose their net charge, the resistance increases and the current decreases (E = IR).
The rate at which the current decreases levels off as the system approaches equilibrium. The current will start to rise again when the pH gradient starts to break down.
IEF needs to be discontinued before this point.

LECTURE 8. «General principles of nucleic acid isolation».

1. NUCLEIC ACID ISOLATION
2. Three major types of techniques
3. Isolation of High Molecular Weight Genomic DNA
4. Plasmid Minipreps and Adsorption Methods.
5. ISOLATION OF RNA
6. Three major types of techniques

, or combinations of them, are employed in the isolation of nucleic acids:
differential solubility,
absorption methods, or
density gradient centrifugation.
The choice of method will depend on the type of DNA being isolated (Box) and the application. A major goal of nucleic acid isolation is the removal of proteins.
The separation of nucleic acids from proteins is generally easily accomplished due to their different chemical properties. In particular, the highly charged phosphate backbone makes the nucleic acids rather hydrophilic as compared to proteins which are more hydrophobic. Separating the different types of nucleic acids can be more problematic in that they all have similar chemistries.
On the other hand, though, this similar chemistry results in a few basic procedures which are common to many nucleic acid isolation protocols.
Most nucleic acid isolation protocols involve a cell lysis step, enzymatic treatments,
differential solubility (eg., phenol extraction or absorption to a solid support), and precipitation.

Genomic (chromosomal)
• Organellar (satellite)
• Phage/Viral (ds or ss)
• Plasmid (extrachromosomal)
• Complementary (mRNA)

Cell Lysis. Nucleic acids must be solubilized from cells or other biological material. This solubilization is usually carried out under denaturing conditions such as: SDS, alkali, boiling or chaotropic agents.
These denaturing conditions efficiently
solubilize the nucleic acids and generally do not adversely affect them.
In addition, the denaturing conditions promote the removal of proteins during the subsequent steps and inhibit the activity of nucleases which will degrade the nucleic acids.

Cell Lysis

1. ± Enzyme Treatment
2. Phenol Extraction or
3. Adsorption Methods
4. EtOH Precipitation

Enzymatic Treatment.

Another approach in the isolation of nucleic acids is to degrade unwanted components.
For example, inclusion of proteases (eg., proteinase K) in the lysate will
promote the removal of proteins. Proteinase K is still active at 55o in the presence of 0.5% SDS.
The elevated temperature and SDS improve solubility and inhibit any DNAse activity that may be present in the lysate.
Nucleases can also be used to remove unwanted nucleic acids. For
example, many DNA extraction protocols include a RNase treatment step, and visa versa. It is important that the RNase be free of DNase activity.
DNase-free RNase is easily prepared by boiling commercial RNase for 10 minutes. The stability of RNase makes the preparation of RNase-free DNase more difficult.
RNase-free DNase should be purchased from a reliable vendor or tested before it use.

- Basic Protocol
- Most DNA extraction protocols consist of two parts
    1. A technique to lyse the cells gently and solubilize the DNA
    2. Enzymatic or chemical methods to remove contaminating proteins, RNA, or macromolecules
- In plants, the nucleus is protected within a nuclear membrane which is surrounded by a cell membrane and a cell wall. Four steps are used to remove and purify the DNA from the rest of the cell.
    1. Lysis
    2. Precipitation
    3. Wash
    4. Suspension

Lysis: grind in Liquid N2 and use detergent
Precipitation Part I: phenol/chloroform extraction to get rid of proteins
Precipitation Part II: addition of salts to interrupt hydrogen bonding between water and phosphates on the DNA
Precipitation Part III: addition of ethanol to pull DNA out of solution
Wash and resuspend: DNA is washed in ethanol, dried, and resuspended in H20 or TE buffer.

LYSIS:
In DNA extraction from plants,
this step commonly refers to the breaking
of the cell wall and cellular membranes (most importantly, the plasma and nuclear membranes)
The cell wall (made of cellulose) is disrupted by mechanical force (for example, grinding the leaves)
Then the addition of a detergent in which breaks down the cell membranes

Detergents can disrupt membranes due to the amphipathic (having both hydrophilic and hydrophobic regions) nature of both cellular membranes and detergent molecules. The detergent molecules are able to pull apart the membranes

The end result of LYSIS is that the contents of the plant cells are distributed in solution.
Phenol extraction

Phenol is an organic solvent that is used to separate proteins from nucleic acids. Proteins are hydrophobic and partition in the organic phase.
Nucleic acids are highly charged and partition in the aqueous phase.

The advantages are of phenol extraction are that it is easy to carry out and can be adapted to many applications. It is also easily applied over a wide range of volumes (40 mkl to several ml). In particular, phenol extraction is widely used for the isolation of high molecular weight genomic DNA.

Phenol extraction is accomplished by mixing the sample with an equal volume of phenol which has been previously saturated with a Tris buffer at pH 8 containing EDTA and NaCl.
The phenol should be molecular biology grade phenol should and store at -20o until preparing the saturated solution.
The saturated solution is stored at 4o. Phenol is easily oxidized, as evidenced by yellowing, and the oxidation products can break DNA.
Oxidized phenol should be discarded.
Depending on the application, the two phases are completely mixed by vortexing, or gently mixed (eg., high molecular weight DNA).
The phases are separated by centrifugation and the upper aqueous phase, which contains the nucleic acids, is retained.
Proteins will often be visible as flocculent material at the top of the phenol phase.
The two phases need to be carefully separated in that the nucleic acids and proteins tend to be at the interface. Leaving too much of the aqueous layer behind will lead to undue loss of material and aspirating too close to the interface can include protein.
The aqueous phase can be re-extracted with phenol to <u>remove more protein.</u>
A common variation of phenol extraction is a mixture of phenol:chloroform: isoamyl

alcohol (25:24:1). The more organic chloroform removes lipids, denatures more protein and mixes less with the aqueous phase leading to more efficient extraction.

Ethanol precipitation

Nucleic acids can be precipitated from dilute solutions with ethanol. This precipitation can be a concentration step or a means to change buffers, especially after phenol extraction.

Typically either sodium acetate or potassium acetate, pH 5.0-5.5, is added to a final concentration of approximately 0.3 M.

The sodium and acidic pH will neutralize the highly charged phosphate backbone and to promote hydrophobic interactions.

Two-to-two and a half volumes of ethanol are added and the sample is incubated as -20o.

If the nucleic acids are small in size and/or in low concentrations an extended incubation (several hours to overnight) is needed.

The precipitated DNA is collected by centrifugation. The pellet is rinsed with 70% ethanol to remove any excess salt, dried and dissolved in the appropriate buffer.

A variation is to substitute ammonium acetate if the 'hard' salts are a problem. Another modification is to use an equal volume of isopropanol (instead of 2-2½ volumes of ethanol) which minimizes the increase in sample volume.

Isolation of high molecular weight genomic DNA

High molecular weight chromosomal DNA is usually isolated by multiple rounds of phenol extraction and enzyme treatments as discussed above.

Shear forces, which can break long DNA molecules, need to be avoided during all steps and samples should never be vortexed.

Therefore, the phenol extraction is carried with gentle rocking for several hours.

Isolation of High Molecular Weight Genomic DNA

These precautions against shear forces are not necessary in the isolation of *low molecular weight DNA*.

Another common modification at the ethanol precipitation step is ‘spool out’ the high molecular weight genomic DNA on the end of a sealed Pastuer pipet.

The precipitated DNA is wrapped *(Завернутый)* around the end of the pipet is then allowed to partially dry and then dissolved in the appropriate buffer.

This minimizes the contamination with RNA and low molecular weight DNA fragments.

- Plasmid Minipreps (*минипродукты*)and Adsorption Methods

Historically, phenol extractions were used for the isolation of most forms of nucleic acids. It is now more common to use techniques based upon adsorption chromatography for the isolation of smaller DNA molecules, such as plasmids. Various kits are available for the rapid isolation of small quantities of plasmid DNA.

The procedure consists of solubilizing the bacteria in an alkali solution followed by neutralization with sodium acetate. The neutralization results in the precipitation of some of the protein and the genomic DNA which is removed by centrifugation.

The soluble material is then mixed with a resin (*Смолы*) in the presence of chaotropic agents (usually guanidine hydrochloride). The resins are usually either based on silica or diatomaceous earth. Under these conditions DNA binds to the matrix, but proteins and RNA do not. The DNA is eluted in a low salt buffer. These methods are rapid and yield a highly purified plasmid DNA which can generally be used directly in most applications without further processing.

Another common application for an adsorption method is the isolation of DNA fragments following gel electrophoresis. In this case the agarose gel piece containing the DNA is dissolved in NaI, *a chaotropic salt*, and the DNA adsorbed to silica (*Кремнезем*).

The DNA is then eluted with a low salt buffer and sometimes gentle heating.

LECTURE 9. Methods of nucleic acid structure analysis. Hybridization and nucleic acids various blotting techniques. Method of Southern blotting. The Northern blotting method. DNA microarray. Reverse northern blotting. RNA-seq (Next-Gen sequencing).

1. Nucleic acid structure analysis

UV Spectrophotometry: Measures absorbance at 260 nm to quantify DNA/RNA and monitor purity.

Circular Dichroism (CD) Spectroscopy: Detects conformational changes in nucleic acids (A-, B-, Z-DNA).

NMR Spectroscopy: Provides atomic-level structural information, particularly for small oligonucleotides.

X-ray Crystallography: High-resolution 3D structure determination of nucleic acid crystals.

Cryo-Electron Microscopy (Cryo-EM): Visualization of large nucleic acid–protein complexes in near-native states.

Atomic Force Microscopy (AFM): Topographical imaging of DNA/RNA molecules on surfaces.

2. Hybridization Principles is based on Watson–Crick base pairing.

Used to detect complementary sequences via labeled probes (radioactive, fluorescent, enzymatic).

Foundation for blotting techniques and microarray technologies.

3. Blotting Techniques

a) Southern Blotting (DNA Detection). Developed by Edwin Southern (1975).

Steps of Southern Blotting:

1. DNA digestion by restriction enzymes.

2. Separation via gel electrophoresis.
3. Transfer to a nitrocellulose/nylon membrane.
4. Hybridization with labeled DNA probe.
5. Visualization (autoradiography or chemiluminescence).

Applications: Gene mapping, detection of mutations, transgene integration.

b) Northern Blotting (RNA Detection)

Detects RNA transcripts.

Steps of Northern Blotting (RNA Detection). Like Southern blot, but RNA is electrophLikeer denaturing conditions, transferred, and probed with complementary DNA/RNA.

Applications: Gene expression studies, transcript size analysis.

c) Western Blotting (Protein Detection) ***(not asked but related)***

Often mentioned alongside Southern/Northern.

Detects proteins using antibodies.

Advanced Hybridization-Based Methods

DNA Microarray

Solid-surface arrays with thousands of DNA probes.

Labeled cDNA/cRNA samples hybridize to complementary spots.

Fluorescence detection used for **transcriptome profiling**, genotyping, SNP analysis.

Powerful but gradually replaced by RNA-seq.

Reverse Northern Blotting

Variation of Northern blot.

Instead of RNA on the membrane, immobilized DNA fragments (genes, cDNA clones) are hybridized with labeled RNA probes.

Applications: Screening gene expression profiles across tissues or conditions.

Next-Generation Sequencing (RNA-seq)

RNA-seq: Sequencing of cDNA generated from RNA.

In summary:

Southern blot → DNA detection.
Northern blot → RNA detection.
Reverse Northern → DNA spotted, probed with RNA.
Microarrays → High-throughput hybridization-based expression profiling.
RNA-seq → Sequence-based, quantitative, comprehensive transcriptome analysis.

LECTURE 10. «Methods of study genes expression»

This involves a promoter sequence being linked to a detectable reporter gene such as luciferase, β-galactosidase or β-glucuronidase. Examples of methods used to determine the expressed reporter gene protein are fluorescence, absorbance and luminescence.

Northern blotting. Western blotting

This is a technique used to detect specific RNA molecules present within an RNA mixture. Northern blotting is employed in the analysis of an RNA sample from a cell type or tissue so as to determine the RNA expression of certain genes.

Western blotting is a technique for detecting specific protein molecules within a protein mixture. This mixture might include all the proteins that are associated with a certain cell type or tissue. The technique can help to determine a protein's size, and how much of it is expressed.

NORTHERN BLOT

How can amounts of RNA be quantified?

The slide shows a virtual Northern with two lanes, one with RNA from control cells, the other with RNA from the experimental sample (eg drug treated cells). Let's say that there is 10x the amount of signal in the experimental sample compared to the control sample for the target gene.

This could mean expression of the gene has increased 10-fold, or it could mean that there is 10x as much RNA in the experimental sample.

To check for this one usually does a so-called 'loading control' in which the blot is probed for expression of a gene which does not change (e.g. actin, GAPDH, cyclophilin, RPLP0 mRNAs; ribosomaL RNA).

In this case, let's say that the loading control shows that there is twice as much RNA in the expt lane.

Thus the real change in the target gene is 10/2 =5 fold. We can express this in a
more general fashion:

Northern blot

NORTHERN BLOT

1. mRNA isolation and purification
2. electrophorese on a gel
3. The gel is probed by hybridizing with a labeled clone for the gene under study.

Fluorescent in situ hybridization (FISH)

This is a cytogenetic technique that can be used to identify and locate specific gene sequences. FISH can be used to visualize copy number aberrations such as the deletion, translocation or amplification of chromosomes. The technique is used in prenatal diagnosis and also provides a useful tool in the diagnosis and predicted prognosis of various sarcomas. The technique is also used in dermatology to help evaluate atypical moles.

Methods for the Study of Gene Expression

Single Transcripts:

- Northern blot
- RNAse protection assay
- Reverse Transcription (RT)-PCR
- Real-time PCR (qPCR)
- In-situ-hybridization
-

Multiple Transcripts Simultaneously:

Dot-Blot analysis

- Differential Display, SAGE
- Subtractive hybridization (1996)
- DNA Microarrays (1999)
- NanoString
- Second generation Sequencing (NGS)

Reverse transcription polymerase chain reaction (RT-PCR)

This is the most **sensitive** technique available for detecting and quantifying mRNA. Using RT-PCR, extremely small sample sizes can be used in the quantification of mRNA and the technique can in fact do this using just a single cell.

LECTURE 11. Molecular markers. Types. Applications in biotechnology. Use of DNA markers in molecular breeding

Genetic markers are used for labeling and tracking the genetic variations in DNA samples.

Genetic markers are biological compounds which can be determined by **allelic variations** and can be used **as experimental probes or labels** to track an individual, tissue, cell, nucleus, chromosomes or genes.

The use of DNA markers in plant and animal breeding has opened new territory in agriculture which is called **molecular breeding**.

These markers are widely used because of their high prevalence and expression in different stages of the organisms.

These markers come from **different classes of DNA mutations:**

1. **such as substitution mutations (point mutations),**
2. **re-assortments (insertions and deletions),**
3. **replication errors and DNA tandem repeats**

These markers are selectively neutral because they are usually **located in non-coding regions of DNA.**

Unlike morphological and biochemical markers, DNA markers are practically unlimited in number and **are not affected by environmental factors and/or the developmental stage of the plant.**

Apart from the use of DNA markers in construction of linkage maps, they have numerous applications in plant breeding such as:

1. assessing the level of genetic diversity within cultivars and
2. fifingerprinting the germplasms.

Many agriculturally important traits such as productivity and quality, tolerance to environmental stresses, and some of forms of disease resistance are quantitative, are controlled by **polygenes** which complicate the breeding process since phenotypic performances only partially reflects the genetic values of individuals. These complex traits are referred to **as quantitative traits** (also called as **polygenic or multifactorial traits**) and the regions within genomes that contain genes associated with a particular quantitative trait are known *as quantitative trait loci (QTLs).*

(also called polygenic, continuous, multifactorial, or complex traits) in nature.

- The genetic variation of a quantitative trait is controlled by the collective effects of numerous genes, known **as quantitative trait loci (QTLs).**

Identification of **QTLs of agronomic importance** and its utilization in a crop improvement requires mapping of these QTLs in the genome of crop species using molecular markers.

Identification of QTLs with genetically linked DNA-markers is useful for incorporating genes into improved cultivars via marker-assisted selection (MAS), map-based cloning of the tagged genes, and for a better understanding of the genetics of complex traits.

Linkage analysis and association

mapping are the two most commonly used methods for QTL mapping. The main items include the two QTL mapping methods with special focus on
mapping population types, linkage map construction, and marker–trait association analysis using different statistical methods and software programs.

- A **quantitative trait locus** (**QTL**)
- is a region of **DNA** that is associated with a particular **phenotypic trait.**
- These QTLs are often found on different **chromosomes**.
- Knowing the number of QTLs that explains variation in the phenotypic trait tells us about the **genetic architecture** of a trait. It may tell us that plant height is controlled by ***many genes*** of small effect, or by a few genes of large effect.
- Another use of QTLs is to identify **candidate genes** underlying a trait.
- Once a region of DNA is identified as contributing to a phenotype, it can be **sequenced.** The DNA sequence of any genes in this region can then be compared to a database of DNA for genes whose function is already known.
- In a recent development, classical QTL analyses are combined with gene expression profiling i.e. by **DNA microarrays**.
- Such **expression QTLs (eQTLs)** describe **cis**- and **trans**-controlling elements for the expression of often disease-associated genes. Observed **epistatic effects** have been found beneficial to identify the gene responsible by a cross-validation of genes within the interacting loci with metabolic pathway- and scientific literature databases
- Principle of QTL Analysis

Identifying a QTL or a gene within a plant genome is like finding the proverbial
needle in a haystack. However, QTL analysis can be used to divide the haystack in
manageable piles *(стог сена в управляемые сваи)* and systematically search them.
In simple terms, QTL analysis is based on the principle of detecting an association between phenotype and the genotype of markers.
Markers are used to partition the mapping population into different genotypic groups based on the presence or absence of a particular marker locus and to determine whether significant differences exist between groups with respect to the trait being measured .
A significant difference between phenotypic means of the groups (either 2 or 3), depending on the marker system and type of population, indicates that the marker locus being used to partition the mapping population is linked to a QTL controlling the trait.

- Traditional QTL Mapping (Linkage Mapping)

The general steps involved in a traditional QTL mapping experiment are as follows:

1. select two parental strains that have differences between them in the alleles that affect variation in a trait. The parents need not be different in the mean phenotypic value of the trait as different allelic combinations can yield the same phenotypic mean;

(2) develop an appropriate mapping population by crossing the selected
parents;
(3) phenotype the mapping population for the trait(s) of interest (morphological characters, agronomic traits, disease and pest scores, drought resistance, etc.) under greenhouse, screen-house, and/or field conditions;
(4) generate the molecular data on the population with adequate number of uniformly spaced polymorphic markers;
(5) construct a genetic map; and
(6) identify molecular markers linked to the trait(s) of interest using statistical programs.

- Mapping Populations Used in QTL Mapping Experiments
- Various types of mapping population may be produced from the heterozygous F1

hybrids:
1. **Double haploid lines (DHLs):** plants are regenerated from pollen (which is
haploid) of the F1 plants and treated to restore diploid condition in which every
locus is homozygous. Since the pollen population has been generated by
meiosis, the DHLs represent a direct sample of the segregating gametes.
2. **Backcross (BC) population**: the F1 plants are backcrossed to one of the parents.
3. **F2 population: F1 plants are selfed.**

- F2:3/F2:4 lines: F3/4 plants tracing back to the same F2 plant, also called F2 families.
- 5. **Recombinant inbred lines (RILs):** inbred generation derived by selfing individual F2 plants and further single seed descent.
- A population of RILs represents an ‘immortal’ or permanent mapping population.

Each of the above mapping populations
(**Double haploid lines (DHLs),**
F2 population: F1 plants are selfed,
Recombinant inbred lines (RILs), possesses advantages and disadvantages.

Hence, the choice of the type of mapping population depends on many factors such as the plant species, type of marker system used, and the trait to be mapped later on. F2 populations, derived from F1 hybrids, and BC populations, derived by crossing the F1 hybrid to one of the parents, are the simplest types of mapping populations developed for self-pollinating species.
Their main advantages are that they are easy to construct and require only a short time to produce.
However, such populations are not fixable due to their inherent heterozygous genetic constitution. This restricts their wide utility in QTL analysis.
The length of time needed for producing RIL population is the major disadvantage, because usually six to eight generations are required.
Development of a DH population takes much less time than RIL; however, the production of DH populations is only possible in species that are amenable to tissue culture (e.g., cereal species such as rice, barley, and wheat).
The major advantages of RIL and DH populations are that they produce homozygous or 'true-breeding' lines that can be multiplied and reproduced without genetic change occurring.
This allows for the conduct of replicated trials across different locations and years. Furthermore, seed from individual RIL or DH lines may be transferred between different laboratories for further linkage analysis and the addition of markers to existing maps. Information provided by co-dominant markers is best exploited by an F2 population, while information obtained by dominant marker systems can be maximized by using RILs or DHLs.
Double haploids, F2 or F3 families, or RILs are advantageous if the trait to be mapped cannot be accurately measured on a single-plant basis but must be assessed in replicated field experiments.
The frequency of genetic information obtained from genetic markers have significant impact on the evolutionary biology, **particularly in understanding the genetic basis of complex traits.**

N**ew concepts, such as quantitative trait loci (QTL)**
mapping followed by the development of statistical tools, have emerged in **quantitative genetics** to identify the genes involved in the genetic variability of complex traits. The complexity of these traits is influenced by the segregation of alleles at many loci, environmental factors and their interactions.

Quantitative Trait Locus (QTL).
Definition. A Quantitative Trait Locus (QTL) is a region of the genome that contributes to the variation of a quantitative trait (e.g., yield, height, drought tolerance, grain quality).
Quantitative traits are controlled by multiple genes and influenced by the environment.

Key features of QTL
Polygenic control: Unlike Mendelian traits, QTLs represent many genes of small effect.
Statistical association: Identified through correlation between **molecular markers** (e.g., SSR, SNPs) and trait variation in mapping populations.
Mapping populations: RILs (Recombinant Inbred Lines), DH (Doubled Haploids), F2, backcrosses used to detect QTLs.
Methods of QTL Mapping
Phenotyping: Measure trait values in a population.
Genotyping: Use DNA markers (SSR, SNPs, AFLP, GBS).
Linkage analysis: Compare marker segregation with trait values.
Statistical models: Interval mapping, composite interval mapping, GWAS (Genome-Wide Association Studies).

Applications
Crop improvement: Identify genomic regions for yield, stress tolerance, disease resistance.
Marker-assisted selection (MAS): Use markers linked to QTLs for early breeding selection.
Gene discovery: Fine-mapping QTLs can lead to identification of causal genes.
Genomic selection: Combine multiple QTLs for predicting breeding values.

LECTURE 11. Molecular markers. Types. Applications in biotechnology. Principles of marker assisted selection (MAS) in crop plants.

1. Definition. Molecular markers are DNA sequences with known locations on the genome that can be used to identify individuals, genotypes, or alleles. They are **heritable, polymorphic, and not influenced by environment**, making them reliable tools for genetic analysis and breeding.
2. Types of molecular markers
a) Hybridization-based
RFLP (Restriction Fragment Length Polymorphism)
Based on variation in restriction enzyme cut sites.
Co-dominant, reliable but labor-intensive.
b) PCR-based
RAPD (Random Amplified Polymorphic DNA) . Uses random primers to amplify anonymous regions.
Quick, low-cost, but low reproducibility

AFLP (Amplified Fragment Length Polymorphism). Combines restriction digestion + selective PCR amplification. High reproducibility, genome-wide, but technically complex.
SSR (Simple Sequence Repeats / Microsatellites) Tandem repeats (e.g., (CA)n).
Highly polymorphic, co-dominant, locus-specific.
ISSR (Inter-Simple Sequence Repeats). Uses primers complementary to SSR motifs. Highly variable, quick, inexpensive.
SNP (Single Nucleotide Polymorphisms). Single base-pair differences in DNA. Abundant across genomes, amenable to high-throughput genotyping.
CAP (Cleaved amplified polymorphic sequence). PCR amplification followed by restriction digestion to detect SNPs. Sequence-based / High-throughput
EST markers (Expressed sequence tags). DArT (Diversity Arrays Technology)**.** NGS-based markers**:** Genotyping-by-sequencing (GBS), whole-genome resequencing.
3. Applications of molecular markers in biotechnology
Genetic diversity analysis: Assess variation among populations or cultivars.
Gene/QTL mapping: Locate genes controlling traits (yield, stress resistance, quality).
Marker-assisted selection (MAS)**:** Accelerate breeding by selecting individuals carrying desirable alleles.
Molecular diagnostics**:** Detect disease resistance genes or transgene presence.
Phylogenetic studies: Clarify evolutionary relationships.
Genetic fingerprinting: Variety identification, seed purity testing, patent protection.
Mutant screening**:** Identify mutations induced by radiation, chemical mutagens, or CRISPR edits.

4. **Principles of marker-assisted selection (MAS) in crop plants**
Traditional breeding relies on phenotype, which is influenced by environment and requires long selection cycles.
MAS uses DNA markers linked to target genes/QTLs for selection, allowing breeders to choose desirable genotypes at seedling stage, without waiting for phenotypic expression.
MAS Workflow
1. Identify a molecular marker tightly linked to a target gene/QTL (e.g., drought tolerance, disease resistance).
2. Genotype the breeding population using PCR-based or SNP-based assays.
3. Select individuals carrying favorable alleles before field evaluation.
4. Advance selected lines for crossing and variety development.

Summary:
Molecular markers are DNA-based tools used for genetic analysis.
Types include RFLP, RAPD, AFLP, SSR, ISSR, SNP, CAP, and modern sequencing-based markers.
Applications span genetic diversity studies, diagnostics, QTL mapping, and breeding.
MAS accelerates crop improvement by linking markers to traits, making breeding more efficient and precise.

LECTURE 12. Genetic engineering methods, applications.

1. Definition. Genetic engineering (also called recombinant DNA technology) is the direct manipulation of an organism's genetic material (DNA/RNA) to alter its characteristics in a precise and targeted manner.
2. Major Methods of Genetic Engineering
a) Recombinant DNA Technology
Principle: Insertion of a foreign gene into a host genome using restriction enzymes and DNA ligase.
Steps: Gene isolation → Vector construction (plasmid, bacteriophage, BAC, YAC) → Transformation into host (bacteria, yeast, plants, animals).
Applications: Insulin production (*E. coli* with human insulin gene), transgenic crops.
b) Gene cloning.
Amplification of specific genes using **vectors** (plasmids, cosmids, viral vectors).
Used for gene characterization, protein production, functional studies.
c) PCR-Based Genetic Manipulation
- **Polymerase Chain Reaction (PCR)** and derivatives (RT-PCR, qPCR, mutagenic PCR).
- Enables site-directed mutagenesis, gene editing, amplification of target sequences.

d) Gene Transfer Methods
1. Physical Methods
Microinjection: Direct injection of DNA into cell nucleus.
Biolistics (Gene Gun): DNA-coated particles shot into plant cells.
Electroporation: Electric pulses create pores for DNA uptake.
Liposome-mediated transfer: DNA packed in lipid vesicles.
2. Biological Methods
Agrobacterium tumefaciens–mediated transformation (in plants).
Viral vectors (retroviruses, adenoviruses in animals).
e) CRISPR-Cas Genome Editing

Principle: RNA-guided Cas nuclease makes double-strand breaks at specific genomic sites → repaired by NHEJ or HDR → insertion/deletion/targeted replacement.
Advantages: Highly specific, efficient, inexpensive compared to TALENs or ZFNs.
Applications: Knock-out/knock-in studies, precision breeding, gene therapy.

f) RNA Interference (RNAi)

Uses siRNA or shRNA to silence specific gene expression post-transcriptionally.
Applied in functional genomics, pest-resistant crops, therapeutic gene silencing.

g) Synthetic Biology & De Novo Gene Synthesis

Entire genes or pathways can be chemically synthesized and inserted into organisms.
Enables creation of **synthetic organisms**, metabolic pathway engineering, biofuel production.

3. Applications of Genetic Engineering

In medicine, in Agriculture, in Industry and environment

Summary:

- **Methods**: Recombinant DNA technology, gene cloning, PCR-based mutagenesis, physical/biological gene transfer, CRISPR, RNAi, synthetic biology.
- **Applications**: Medicine (therapeutics, gene therapy), Agriculture (transgenic crops, biofortification), Industry/Environment (biofactories, bioremediation).

LECTURE 13. Omics driven bioengineering. Metabolic engineering.

Omics-Driven Bioengineering.

1. Concept. Integrates omics technologies (genomics, transcriptomics, proteomics, metabolomics, epigenomics, phenomics) to guide rational engineering of biological systems.
Enables a systems biology approach: not only focusing on a single gene/protein, but network-wide regulation of traits.
2. Key Omics Contributions.
Genomics: Whole-genome sequencing, gene discovery, SNP identification for target traits.
Transcriptomics (RNA-seq, scRNA-seq): Gene expression profiles under stress, growth, or production conditions.
Proteomics (LC-MS/MS, quantitative MS): Post-translational modifications, enzyme abundances, signaling pathways.
Metabolomics (GC-MS, LC-MS, NMR): Identifies metabolic bottlenecks, fluxes, accumulation of intermediates.
Epigenomics: DNA methylation, histone modifications affecting gene regulation.
Phenomics: High-throughput phenotype analysis linked to genotypes and metabolic traits.
3. Applications in bioengineering
Microbial cell factories: Improving *E. coli*, *Saccharomyces*, *Corynebacterium* for bioproduction.
Crop improvement: Integrating omics to engineer drought tolerance, nutrient enrichment, disease resistance.
Synthetic biology: Designing synthetic pathways using omics-guided metabolic maps.
Precision medicine: Omics-based biomarkers guide targeted gene editing or therapies.
Metabolic Engineering.
1. Definition. Metabolic engineering = redesigning metabolic pathways in organisms (microbes, plants, animals) to enhance the production of desired compounds or introduce new biosynthetic abilities.
2. Principles
1. Identify pathway: Using omics data and metabolic flux analysis.
2. Modify genes/enzymes: Overexpress rate-limiting enzymes, knock out competing pathways, introduce heterologous genes.
3. Optimize flux: Redirect carbon, nitrogen, or energy flow toward target product.
4. Systems-level modeling: Genome-scale metabolic models predict optimal interventions.
3. Engineering Tools
Classical tools:
Recombinant DNA technology (gene overexpression/knockout).
Mutagenesis (random or targeted).
Modern tools:
CRISPR-Cas: Precision editing of metabolic genes.
Synthetic biology: Modular design of new pathways.
Adaptive laboratory evolution (ALE): Evolving strains under selective pressures.
Flux balance analysis (FBA): Computational modeling of metabolic networks.
4. Applications
Pharmaceuticals: Production of antibiotics (erythromycin, penicillin), artemisinin (antimalarial), taxol.
Food and nutrition: Vitamin production (Vitamin B12, folate), amino acids (lysine, tryptophan).
Biofortification in crops (iron, zinc, provitamin A).
Biofuels:
Ethanol, butanol, biodiesel from engineered microbes.

Industrial Biotechnology:
Biopolymers (PHA, PLA), organic acids (lactic acid, succinic acid).
Environmental:
Engineering microbes/plants for carbon sequestration, bioremediation.

Omics + Metabolic Engineering = Systems Metabolic Engineering

Omics data identify key bottlenecks.
Metabolic models predict engineering targets.
Genetic tools (CRISPR, RNAi, synthetic biology) implement modifications.
Adaptive evolution fine-tunes strains.

Example:

Artemisinin production in *Saccharomyces cerevisiae*:
Omics-guided identification of flux bottlenecks → engineered mevalonate pathway → high-yield artemisinic acid → converted to artemisinin (antimalarial drug).

Summary:

Omics-driven bioengineering integrates system-wide data for rational design.

Metabolic engineering modifies metabolic pathways to enhance production.

Together, they form systems metabolic engineering – driving innovations in medicine, agriculture, energy, and environment.

LECTURE 14. RNA interference (RNAi) method applied in genetic engineering. Synthetic biology and de novo gene synthesis.

RNA interference (RNAi) is post-transcriptional gene silencing by small RNAs (21–24 nt) guiding RISC to cleave or repress target mRNAs.

Main plant implementations

hpRNA (hairpin RNA / ihpRNA): Invert a target fragment (≈200–500 bp) separated by an intron spacer under a strong promoter (e.g., maize Ubiquitin, CaMV 35S for tissues where it's active, or tissue/inducible promoters).

amiRNA: Re-engineer an endogenous miRNA precursor to carry a 21-nt sequence against your gene; highly specific; great for single-gene knockdowns.

VIGS (virus-induced gene silencing): Fast, transient; clone 200–300 bp into vectors like BSMV/TMV; ideal for quick screening in leaves (e.g., LR assays).

`HIGS (host-induced gene silencing): Plant expresses siRNAs against pathogen genes (e.g., essential Puccinia transcripts) to reduce virulence.

Design steps (for TaVIT2, TaZIFL, ZTP29, etc.)

1. Target region choice
    a. Prefer coding sequence; avoid UTRs with isoform variability.
    b. Exclude regions with SNP hot spots across homeologs unless you want pan-homeolog knockdown.
2. Off-target control
    a. BLAST against the wheat genome; require ≥3 mismatches across the seed (nt 2–8).
    b. For homeolog-specific silencing, place ≥1 mismatch at positions 10–11 of the guide.
3. Construct
    a. hpRNA: 250–400 bp sense–intron–antisense; intron spacers like PIV2 or Wrab17 introns work well.
    b. amiRNA: use a wheat miR319 or miR171 backbone; design 21-nt guide with correct star/passenger pairing and mismatches at positions 1 and 12.
4. Promoter strategy
    a. Leaf rust assays: leaf/mesophyll-active (Rubisco small subunit) or pathogen-inducible promoters (e.g., PR-1-like) to minimize pleiotropy.

LECTURE 15. Cell engineering. Tissue engineeringю

Definition. Cell engineering is a field of biotechnology that deals with the modification, manipulation, and use of cells to solve fundamental and applied problems.

Main areas

• Genetic modification of cells (introduction or deactivation of genes: CRISPR/Cas, transfection, transduction).
• *In vitro* cell culture (animal, plant, microbial).
• Creation of cell lines with specific properties (e.g., stress-resistant, protein, hormone, or antibody producers).

Bioreactors for cells: large-scale production (insulin, monoclonal antibodies, vaccines, secondary plant metabolites).
Examples of applications
Medicine: CAR-T therapy (modification of T lymphocytes against cancer).
Plants: creation of somatic hybrids, cell cultures for the synthesis of alkaloids/vitamins.
Industry: cell factories (yeast, bacteria, CHO cells).

Tissue Engineering

Definition. Tissue engineering is an interdisciplinary field (biology, materials science, medicine) whose goal is to create or restore tissues and organs using cells, biomaterials, and bioreactors.
Key elements
1. Cells (stem cells, differentiated cells, induced iPSCs).
2. Matrices/scaffolds (biocompatible scaffolds — polymers, hydrogels, collagen).
3. Growth signals (growth factors, biochemical stimuli, mechanical loads).
Examples of application
• In medicine: artificial skin, cartilage, bone tissue, vascular implants.
• In pharmacy: organ-on-chip tissue models for drug testing.
• In food biotechnology: "artificial meat" (cultured meat).
Comparison of Cell vs Tissue Engineering

1. Hybridization-Based Methods

Northern blotting

Detects specific RNA transcripts.
Provides information about transcript size and abundance.
Limitations: Low sensitivity, labor-intensive, requires high-quality RNA.

Reverse Northern blotting

DNA/cDNA immobilized, probed with labeled RNA.
Useful for comparing expression across tissues/conditions.

DNA Microarrays

Thousands of probes spotted on a chip.
Fluorescently labeled cDNA from RNA samples hybridizes to complementary probes.
Applications: Genome-wide expression profiling, SNP detection.
Limitations: Requires known sequences, lower sensitivity than RNA-seq.

2. PCR-Based Methods

- **RT-PCR (Reverse Transcription PCR)**

Converts RNA → cDNA, then amplifies specific genes.
Detects presence/absence of gene expression.

qPCR (Real-Time Quantitative PCR)

Measures amplification in real time using fluorescent dyes/probes (SYBR Green, TaqMan).

3. Sequencing-based methods

RNA-seq (Next-Generation sequencing)

Direct sequencing of cDNA libraries.
Provides quantitative and qualitative data: expression levels, isoforms, splicing, novel transcripts.
Advantages: High sensitivity, unbiased, genome-wide.
Limitations: Costly, requires computational resources.

- **Single-cell RNA-seq (scRNA-seq)**

Expression profiling at the single-cell level.
Reveals cell-to-cell heterogeneity and rare populations.

4. In Situ Hybridization (ISH)

Uses labeled RNA/DNA probes to detect transcripts **within intact tissues or cells**.
Fluorescent ISH (FISH) and **RNAscope** allow high-resolution visualization.
Applications: Spatial localization of gene expression.

5. Reporter gene assays

Fusion of promoter/gene regions to **reporter genes** (GUS, GFP, luciferase).
Reporter activity reflects expression pattern.
Applications: Studying promoter activity, tissue-specific expression.

6. Protein-level expression (Indirect gene expression studies)

Western blotting: Detects protein products of expressed genes.
ELISA: Quantitative protein detection.
Mass spectrometry-based proteomics: High-throughput expression profiling at protein level.

7. Advanced and emerging approaches

Nanostring technology: Digital detection of transcripts without amplification.
CRISPR-based live-cell RNA imaging: Tracks transcripts in real time.
Spatial transcriptomics: Combines RNA-seq with tissue imaging to map gene expression in situ.

Summary:

1. Classical methods: Northern blot, ISH, reporter assays.
2. Quantitative methods: qPCR, RT-PCR.
3. High-throughput: Microarrays, RNA-seq, scRNA-seq, spatial transcriptomics.