Brief content of lecture

Discipline *"Methods of molecular biotechnology"*

Lecture 1. Physical and Structural techniques Nucleic acids

DNA extraction methods

DNA Extraction

- **Purpose of DNA extraction**
- **Review the main steps in**

the DNA extraction protocol

and the chemistry

Purpose of DNA Extraction

To obtain DNA in a relatively purified form which can be used for further investigations, i.e. PCR, sequencing, etc

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. **Resuspension**

A comparison of DNA extraction methods used in research labs as opposed to "classroom labs"

<u>Research</u>

***Lysis*: grind in Liquid N_2 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.**

"Classroom"

***Lysis*: grind in mortar/pestel and use detergent**

***Precipitation Part I*: NONE (chemical are too dangerous!)**

***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 the which breaks down the cell membranes**
    - **Detergents are able to 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.**

Why add detergent?

Blending separated the pea cells, but each cell is surrounded by a sack (the cell membrane). DNA is found inside a second sack (the nucleus) within each cell.

To see the DNA, we have to break open these two sacks.

Why add detergent?

We do this with detergent.

Think about why you use soap to wash dishes or your hands. To remove grease and dirt, right?

Why add detergent?

Soap molecules and grease molecules are made of two parts:

Heads, which like water

Tails, which hate water.

Why add detergent?

Both soap and grease molecules organize themselves in bubbles (spheres) with their heads outside to face the water and their tails inside to hide from the water.

Why add detergent?

When soap comes close to grease, their similar structures cause them to combine, forming a greasy soapy ball.

Why add detergent?

A cell's membranes have two layers of lipid (fat) molecules with proteins going through them.

Why add detergent?

When detergent comes close to the cell, it captures the lipids and proteins.

What is an enzyme?

After the detergent step, the last question was: what do you have now in your pea soup?

The cell and nuclear membranes have been broken apart,
as well as all of the organelle membranes.

What is an enzyme?

The DNA in the nucleus of the cell is molded, folded, and protected by proteins. The meat tenderizer cuts the proteins away from the DNA.

Extracting DNA

DNA will rise into the alcohol layer from the pea layer

Use a wooden stick draw DNA into the alcohol

What is the stringy stuff?

Alcohol is less dense than water, so it floats on top.

Since two separate layers are formed, all of the grease and the protein that we broke up in the first two steps and the DNA have to decide which layer to go to.

What is the stringy stuff?

In this case, the protein and grease parts find the bottom, watery layer the most comfortable place, while the DNA prefers the top, alcohol layer.

DNA is a long, stringy molecule that likes to clump together.

What is the stringy stuff?

DNA is a long, stringy molecule. The salt you added in step one helps it stick together. So what you see are clumps of tangled DNA molecules!

DNA normally stays dissolved in water, but when salty DNA comes in contact with alcohol it becomes undissolved. This is called precipitation. The physical force of the DNA clumping together as it precipitates pulls more strands along with it as it rises into the alcohol.

You can use a wooden stick or a straw to collect the DNA. If you want to save your DNA, you can transfer it to a small container filled with alcohol.

PRECIPITATION (In a lab):
This a series of steps where DNA is separated from the rest of the cellular components

In a research lab, the first part of precipitation uses phenol/chloroform to remove the proteins from the DNA

Phenol denatures proteins and dissolves denatured proteins.

Chloroform is also a protein denaturant

THIS STEP CANNOT BE PERFORMED IN "CLASSROOM LABS"!!

The second part of research lab DNA precipitation is the addition of salts

The salts interrupt the hydrogen bonds between the water and DNA molecules.

The DNA is then precipitated from the protein in a subsequent step with isopropanol or ethanol

In the presence of cations, ethanol induces a structural change in DNA molecules that causes them to aggregate and precipitate out of solution.

The DNA is pelleted by spinning with a centrifuge and the supernatant removed

PRECIPITATION (In a "classroom lab"):
This a series of steps where DNA is separated from the rest of the cellular components

In a classroom lab, DNA precipitation involves the addition of salts

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Note: because this protocol does not use phenol/chloroform, the DNA extracted in a classroom lab is not as "clean" as the DNA extracted in a research lab!

Washing:

The precipitated DNA is laden with acetate salts. It is "washed" with a 70% ethanol solution to remove salts and other water soluble impurities but not resuspend the DNA.

Resuspension:

The clean DNA is now resuspended in a buffer to ensure stability and long term storage.

The most commonly used buffer for resuspension is called 1xTE

Checking the Quality of your DNA

- **The product of DNA extraction will be used in subsequent experiments**
- **Poor quality DNA will not perform well in PCR**
- **To assess the quality of DNA extraction use the following simple protocol:**
    - **Mix 10 µL of DNA with 10 µL of loading buffer**
    - **Load this mixture into a 1% agarose gel**

▶ **Analyze results (the following slides provide guidance)**

Expected Results in a
"Classroom Lab"

Using the protocol in the Cereal Genomics module, the genomic DNA extracted will look different than the optimized DNA extraction on the previous slide (*this is mainly due to the missing phenol/chloroform step*)

This is expected. Even though this genomic DNA preparation is not perfect, it is suitable for use as a PCR template

Lane A: Barley

Lane B: Corn

Lane C: Oat

Lane D: Rice

Lane E: Wheat

LECTURE 2 Extraction of RNA

1. **Main principes of RNA Extraction**
2. **A Rapid, Quantitative Assay for Direct Detection of MicroRNAs and Other Small RNAs Using Splinted Ligation**

- ISOLATION OF RNA

Most RNA isolation protocols also involve phenol extractions and are similar to DNA isolations.

However, there are some differences and special considerations.

In particular, precautions *(Меры предосторожности)* against RNase activity must be taken.

RNase is an extremely stable and active enzyme.

Gloves should be worn at all times and sterile plastic ware should be used whenever possible to avoid introducing exogenous RNase to the sample.

Glassware needs to treated with DEPC-water and autoclaved to inactivate any RNase. Buffers should be prepared from DEPC-water or RNase inhibitors included.

- RNase inhibitors!
- • extraction in guanidine salts
- • phenol extractions at pH 5-6
- • treatment with RNase-free DNase
- • precipitation with LiCl
- • oligo-dT column

The cell lysis and solubilization of RNA will typically be carried out in guanidine salts (especially guanidine thiocyanate). **Guanidine is a strong chaotropic agent** and will inhibit RNases.

The strong denaturing effect of this salt will also promote better phenol extraction.

The phenol extraction is the same as the DNA isolation except that the phenol is usually saturated with a buffer of pH 5-6.

The lower pH will result in some DNA partitioning in the organic phase.

If DNA contamination is a problem, it is possible to purchase RNase-free DNase. Long term storage is best as a precipitate in 70% ethanol at –20o.

- Precipitation of RNA with LiCl.

LiCl has been used to selectively precipitate RNA.

Large RNAs (rRNA, mRNA) are insoluble at high ionic strength, whereas small RNAs (tRNA

and 5S rRNA) and DNA generally remain soluble. Following either phenol or guanidine

extraction, an equal volume of 8 M LiCl is added. The sample is mixed vigorously and

incubated at -20o.

The precipitate is collected by centrifugation and reprecipitated if necessary.

- Affinity chromatography

Most eukaryotic mRNA contains a stretch of A residues at its 3' end which added post-transcriptionally.

It is possible to isolate mRNA by affinity **chromatography on oligodT columns** (Figure).

The RNA solution is passed

over an oligo-dT column under conditions which promote base pairing.

Only RNA with a polyA tail binds. The polyA RNA is eluted under

conditions (usually low salt and high temperature) which breaks the base pairing.

- DENSITY GRADIENT CENTRIFUGATION

Density gradient centrifugation can also be used in the analysis and isolation of NA.

Double-stranded DNA, single-stranded DNA, RNA and proteins all have different densities in **CsCl**

and therefore can be separated by **isopycnic** (i.e., equilibrium) centrifugation.

- CsCl is the standard medium for the density gradient centrifugation of nucleic acids and are especially useful for the purification of large amounts of highly purified DNA.

The gradients are carried out in the presence of ethidium bromide which fluoresces when bound to DNA. The DNA bands are detected by illumination with ultraviolet light and easily recovered with a syringe and needle by puncturing the wall of the disposable tube and aspirating the DNA.

The CsCl can be removed by dialysis or by precipitating the DNA.

Density in CsCl

- DNA ~ 1.7 g/cm3
- Protein ~ 1.3 g/cm3
- RNA > DNA
- ssDNA > dsDNA

The %G:C content affects the density of DNA (Figure).

This can result in multiple bands on CsCl gradients if DNA composed different **G:C** compositions are centrifuged.

For example, minor bands, called **satellite DNA**, are often observed when total DNA from an organism is analyzed by CsCl gradients.

These **satellite bands** are usually due to highly repetitive DNA or organellar DNA.

Mitochondrial DNA has high **A:T content**.

These satellite DNA bands can be purified from the genomic DNA by *density gradient centrifugation*.

- density (g/cm3)
- Nucleic acids can also be separated according to **size** by *rate zonal centrifugation on sucrose gradients*.
-
- However, this method is not widely used since **gel electrophoresis i**s generally a more convenient method for the size fractionation of nucleic acids.
- A Rapid, Quantitative Assay for Direct Detection
  of MicroRNAs and Other Small RNAs Using Splinted Ligation

(miRNAs) are **endogenous small noncoding RNAs** that regulate gene expression by binding to target messenger RNAs and inducing translational repression, cleavage, or destabilization of the target.

Each miRNA can potentially regulate expression of a distinct set of genes and therefore miRNAs is ideally suited to rapidly adjusting protein concentrations in cells.

miRNAs is required during cell differentiation. In fact, certain miRNAs are differentially expressed, both spatially and temporally, in many types of immune cells.

Moreover, consistent with the discovery that miRNAs modulate gene expression, altered miRNA expression has been associated with various types of diseases,

including cancer.

Northern blotting is the standard method for the detection of small RNAs, because it allows direct comparison of the quantity of small RNA between different samples.

However, major drawbacks (*недостатки*) of Northern blotting are:

1. the time-consuming procedures and,
2. poor sensitivity, especially when monitoring expression of short nucleotide sequences.

3. Northern blotting requires relatively large amounts of starting material and involves multiple handling steps (Table 1).

- Direct Detection of MicroRNAs by ***splinted ligation***

This protocol describes a method that uses ***splinted ligation*** (*шинирование*) for in-solution, direct labeling of small RNAs from total RNA.

In mol. Biology ***ligation*** *is* the covalent linking of two ends of DNA or RNA molecules.

The liquid phase hybridization method makes it possible to achieve sensitive, specific, and quantitative detection while eliminating a number of time-consuming and labor-intensive steps required for the standard **Northern blot assay**.

The assay uses a small **RNA-specific bridge oligonucleotide (**РНК-специфический мостиковый олигонуклеотид to form **base pairs with the small RNA** and a 5′ end radiolabeled ligation oligonucleotide.

The captured (*захваченный*) small RNA is internally labeled by ligation.

Detection of the labeled small RNAs is performed by **denaturing gel electrophoresis** and autoradiography or phosphorimaging.

This protocol has been successfully used to study expression of various classes of biological small RNAs from nanogram to microgram amounts of total RNA **without an amplification step** and is significantly more simple and more sensitive than Northern blotting or ribonuclease protection assays.

Once the oligonucleotides have been synthesized and total RNA has been extracted, the procedure can be completed in 6 h.

- **Table. Comparison of the amplification-independent assays for small RNA detection**

- The splinted-ligation technique

(Fig. 1) is a nucleic acid hybridization assay that uses a bridge oligonucleotide with perfect Watson–Crick complementarity to a target small RNA and a

5/ end radiolabeled ligation oligonucleotide.

Simultaneous base pairing between both the small RNA and ligation oligonucleotide to the bridge oligonucleotide yields a double-stranded structure with a nick on one strand, which can be ligated with T4 DNA

Ligase, thus labeling the target small RNA.

In addition, because the labeled phosphate provided by the ligation oligonucleotide is

- ***The protocol is divided into five steps:***

1, labeling of the ligation oligonucleotide;

2, capturing of the ligation oligonucleotide and small RNA on a bridge oligonucleotide,

3. linking of the ligation oligonucleotide to the small RNA using T4 DNA ligase;

step 4, partial removal of labeled phosphate from the unligated oligonucleotide;

step 5, fractionation on a denaturing gel and detection by a phosphorimager.

The gel image shows detection of *miR-21* by splinted ligation.

Assay reactions were performed with the indicated amounts of HeLa cell total RNA.

Lanes designated “neg” is a no RNA negative control and “pos” is a synthetic *miR-21* positive control.

These controls were complete reactions in which the RNA samples were replaced by water and 2.5

femtomoles synthetic *miR-21* RNA, respectively. *Lane M* is 5¢ end-labeled oligodeoxynucleotides markers. The *top arrow*

indicates the position of *miR-21* ligated to the ligation oligonucleotide. The *bottom arrow* indicates residual radiolabeled

14 nt ligation oligonucleotide that is present due to incomplete removal of the 5¢ end-32P.

- **The gel image of mir-RNA detection by splinted-ligation technique**

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- ***ANALYSIS AND QUANTIFICATION of NA***

The quality and quantity of isolated NA can be determined spectrophotometrically

Nucleic acids have an Amax of 260 nm and proteins have Amax of 280 nm.

The A260/A280 ratio is therefore indicative of the degree of purity of the nucleic acid. A260/A280 ratios

of 1.6-1.8 or 1.8-2.0 are usually acceptable for DNA and RNA, respectively.

The standard extinction coefficient used for ssDNA, dsDNA and RNA are 0.03 ml/µg, 0.02 ml/µg and 0.025

ml/µg, respectively.

Formulas which take into account protein and other contaminants are also available.

Indirect spectrophometric assays for DNA quantification are also available, but rarely used. In some instances fluorometry using fluorescent dyes that bind DNA and/or RNA is used to determine nucleic acid concentrations.

- DNA A260 1.0 ≈ 50 µg/ml A260/A280 1.6 - 1.8
- RNA A260 1.0 ≈ 40 µg/ml A260/A280 ~2.0
- Spectrophotometric Conversions
- 1 A260 unit of double-stranded DNA = 50 µg/ml

- 1 A260 unit of single-stranded DNA = 33 μg/ml
- 1 A260 unit of single-stranded RNA = 40 μg/ml
- **DNA Molar Conversions**
- 1 μg of 1,000 bp DNA = 1.52 pmole (3.03 pmoles of ends)
- 1 pmole of 1,000 bp DNA = 0.66 μg
- **Protein Molar Conversions**
- 100 pmoles of 100,000 dalton protein = 10 μg
- 100 pmoles of 50,000 dalton protein = 5 μg
- 100 pmoles of 10,000 dalton protein = 1 μg
- **LECTURE 3. Hybridization Conditions and Melting Temperature**
- ***Considerations for better oligonucleotide design***
- Hybridization is a common step of many molecular biology protocols.
- Examples of techniques that include nucleic acid hybridization are:

1. northern and Southern analysis,
2. PCR/qPCR,
3. cloning,
4. *in situ* hybridization,
5. array analysis, gene knockdown, and
6. next generation sequencing (NGS).

- **Important Factors That Affect Stringency and Hybridization**
- *Temperature of hybridization and salt concentration*
    - Increasing the hybridization temperature or decreasing the amount of salt in the buffer increases probe specificity and decreases hybridization of the probe to sequences that are not 100% the same.
- *Concentration of the denaturant in the buffer*
    - For example: Deionized Formamide and SDS can be used to reduce non-specific binding of the probe
- *Length and nature of the probe sequence*
- The criteria for hybridization are based on **nucleic acid strand melting**.
- Therefore, an understanding of melting temperature (T_m) provides information on when and how the DNA or RNA strands are going to hybridize and defines the rules for hybridization.
- It is very important to understand this process so that you can better design and optimize the oligos for your experiments.

- **Oligonucleotide concentration**
- T_m varies with oligonucleotide concentration.
- While folding of a single oligonucleotide is concentration independent, the T_m is strongly influenced by oligo concentration when 2 or more nucleic acid strands interact.
- Oligo concentration alone can cause T_m to vary by ±10°C. The strand that is in excess determines T_m.
- For example, in PCR/qPCR, the target concentration is usually designed to be much lower than that of the probe.
- In such situations, T_m is determined by the probe because it is in excess.
- **Salt environment**
- The concentrations of monovalent (sodium, potassium), divalent (magnesium), and polyvalent cations affect the stability of hybridized oligonucleotides.
- Divalent cations have the biggest impact on T_m—changes in the millimolar range are significant. Increasing the concentration of monovalent cations, such as Na^+, up to 1–2 M stabilizes oligos.
- However, these higher concentrations can significantly impact Tm. "A change from 20–30 mM Na^+ to 1 M Na^+ can cause oligonucleotide T_m to vary by as much as 20°C," "We have worked through the calculations for sodium and magnesium ion concentrations and have come up with complicated models to predict T_m.
- **Salt environment**
- This formula is used in the IDT SciTools® programs to ensure your calculations are correct." (See the formula in the above sidebar, *Accurate T_m Calculation*, and read a detailed explanation in the Technical Report, *Calculation of T_m for Oligonucleotide Duplexes*, listed in the Related Reading box below). The free online SciTools programs, such as the OligoAnalyzer® tool, are available on the IDT website at www.idtdna.com/scitools.
- Only free Mg^{2+} in solution reacts with DNA; therefore, it is also important to consider the presence of any compounds that bind magnesium ions.
- **Salt environment**
- For example, PCR reactions require deoxynucleoside triphosphates (dNTPs) in a mixture with short oligomer primers, probes, and longer nucleic acid targets. Magnesium ions bind to all of these components, thus decreasing the concentration of free Mg^{2+}.
- As DNA synthesis proceeds during PCR, dNTPs are incorporated into the products and pyrophosphate is released.
- Pyrophosphate also binds Mg^{2+}. Ideally, all of this should be considered when estimating the concentration of free Mg^{2+} in the hybridization solution.
- ***Mismatches*** Несоответствия ***and single nucleotide polymorphisms (SNPs)***
- Mismatches between hybridizing oligos have a profound (*глубокий*) effect on T_m. The effect depends both on sequence context and solution composition (while salt is the main factor considered, other additives, like urea, DMSO, and even SYBR® Green, can shift T_m).

- A single mismatch can cause T_m to vary between 1 and 18°C in PCR applications. The identity of the mismatch, its position in the sequence, and its context all impact the degree of the mismatches effect. A-A and A-C are among the least stable mismatch pairs, causing the largest T_m variation, as compared to G-T, one of the more stable mismatch pairs.
- The context of the mismatch (e.g., whether a G-T mismatch is adjacent to an A-T or G-C base pair) also affects T_m.
- **Mismatches and single nucleotide polymorphisms (SNPs)**
- SNPs that underlie primer and probe hybridization sites, cause mismatches.
- Thus, "SNPs can affect PCR/qPCR experimental outcomes.
- There are a lot of SNPs out there, and their numbers are growing exponentially!
- Thus, we must design our PCR/qPCR assays intelligently, with SNPs in mind. It is important to check NCBI's dbSNP database (www.ncbi.nlm.nih.gov/snp) to determine whether SNPs have been identified within your amplicon.
- Then you can attempt to design your primer and probe locations around those SNPs, as the mismatches they create could likely affect your PCR results,". "However, by positioning them towards the 5' end of the probe or primer sequences, thus, away from the 3' end, these effects can be minimized or even prevented."
- Stringency is a term that many molecular technologists are all very familiar with. It is a term that describes the combination of conditions under which a target is exposed to the probe. Typically, conditions that exhibit high stringency are more demanding of probe to target complementarity and length. Low stringency conditions are much more forgiving.
- If conditions of stringency are too HIGH → Probe doesn't bind to the target
- If conditions of stringency are too LOW → Probe binds to unrelated targets
- **Melting Temperature (T_m) Long Probes**
- The ideal hybridization conditions are estimated from the calculation of the T_m.
- The T_m of the probe sequence is a way to express the amount of energy required to separate the hybridized strands of a given sequence.
- At the T_m: Half of the sequence is double stranded and half of the sequence is single stranded.
- $Tm = 81.5°C + 16.6\log M + 0.41(\%G+C) – 0.61(\%formamide) – (600/n)$
- Where M = Sodium concentration in mol/L
- n = number of base pairs in smallest duplex
- If we keep in mind that RNA is single stranded (ss) and DNA is double stranded (ds), then the following must be true:
- RNA : DNA Hybrids More stable
- DNA : RNA Hybrids ↓
- DNA : DNA Hybrids Less stable

- T_m of RNA probes is higher, therefore RNA : DNA hybrids increase the Tm by 20 – 25°C
- **Calculating the T_m for Short Probes (14 – 20 base pairs)**
- Tm = 4°C x number of G/C pairs + 2°C x number of A/T pairs
- The hybridization temperature (annealing temp) of oligonucleotide probes is approximately 5°C below the melting temperature.
- **Sequence Complexity ($C_o t$)**
- Sequence complexity refers to the length of unique, non-repetitive nucleotide sequences.
- $C_o t$ = Initial DNA Concentration (C_o) x time required to reanneal it (t)
- $C_o t_{1/2}$ = Time required for half of the double-stranded sequence to anneal under a given set of conditions.
- Short probes can hybridize in 1 – 2 hours, where long probes require more time.

- **LECTURE 4.** MODIFICATION OF NUCLEIC ACIDS
- All DNA molecules are similar in terms of their **biochemical and physical properties.**

Therefore, unlike proteins, DNA techniques are not highly dependent upon the particular gene being studied.

Genomic DNA is an extremely large molecule. For example, the

human genome contains approximately 2 x 109 base pairs (bp).

The size of a gene for a 50 kDa protein might be as small as 2000 bp (or 2 kb). Therefore, it can be quite difficult to identify and characterize specific genes from an organism.

- **The study of specific genes involves**
- manipulating nucleic acids.
- In particular, it is possible to break DNA into smaller fragments, identify fragments of interest and to amplify these fragments so that they can be analyzed.
- Enzymes are used to carry out these manipulations of DNA and RNA. DNA modifying enzymes include:

1. **polymerases,**
2. **ligases and**
3. **nucleases**.

Polymerases synthesize nucleic acids in a template mediated fashion.

Ligases join fragments of DNA.

Nucleases cleave the **phosphodiester bond** between nucleotides. A wide range of specificities are exhibited by nucleases.

Exonucleases remove nucleotides one at a time from either the 5' or 3' end.

In addition, some exonucleases exhibit substrate specificities in terms of preferences for double-stranded (ds) or single-stranded (ss) DNA or RNA.

Endonucleases cleave the phosphodiester bond in the middle of a oligonucleotide and produce fragments.

Such exo- and endonucleases have some applications primarily directed at removing unwanted types of nucleic acids or removing single stranded overhangs from dsDNA.

- RESTRICTION ENDONUCLEASES
- **Three distinct classes of restriction endonucleases, designated types I, II and III:**
- **Type I** cleavage occurs 400-7000 bp from recognition site
- **Type II** cleavage occurs adjacent or within recognition site
- **Type III** cleavage occurs 25-27 bp from recognition site.
- Class II restriction enzymes are the most useful since they cleave DNA at the recognition site.

The recognition sequences of type II restriction enzymes are generally *4-8 consecutive (последовательный)nucleotides.*

Some restriction enzymes do allow for some *degeneracy* (*вырождение*) at a particular nucleotide.

This degeneracy can be restricted to **a purines (R)** or **pyrimidines (Y),** or be completely

unrestricted (N).

Most recognition sites are ***palindromes*** in that both strands exhibit the same sequence.

The sequence complementarity and opposite orientations of the two strands leads to a dyad symmetry.

Hydrolysis of the phosphodiester bond results in the phosphate on the 5'-carbon and the hydroxyl on the 3'-carbon.

Both DNA strands are cut between the same two residues.

This will result in blunt ends if the cleavage site is in the exact center of the recognition site.

Alternatively, 5' overhangs (or extensions) or 3' overhangs of varying length will be

produced if the cleavage site is not in the center of the recognition site.

- **Isoschizomers**
- are restriction enzymes isolated from different sources that recognize ***the same sequence***, but cleave at different positions resulting in different overhangs.
- In addition, different recognition sites can produce identical overhangs (called compatible ends).

LECTURE 5. Main principles of electrophoresis for analysis of nucleic acids

- 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., H_3PO_4).
- 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.
- The pH gradient can be determined with **marker proteins** with known isoelectric points or by measuring the pH along the gel.
- This is accomplished by slicing the gel into pieces,
- eluting the ampholytes into distilled water and measuring the pH.

LECTURE 6. *Nucleic Acid Detection.*

LECTURE 6. NUCLEIC ACID DETECTION

- Autoradiography
- *in situ* hybridization
- DNA Separation Techniques
- PCR and RT-PCR
- 3.5 Nucleic Acid Detection
- Hybridization of a labeled nucleic acid to complementary sequences can identify specific nucleic acids.
- probe – A radioactive nucleic acid, DNA or RNA, used to identify a complementary fragment.
- 3.5 Nucleic Acid Detection
- autoradiography – A method of capturing an image of radioactive materials on film.
- 3.5 Nucleic Acid Detection
- *in situ* hybridization – Hybridization of a probe to intact tissue to locate its complementary strand by autoradiography.
- DNA Separation Techniques
- Gel electrophoresis separates DNA fragments by size, using an electric current to cause the DNA to migrate toward a positive charge.
- DNA Separation Techniques
- DNA can also be isolated using density gradient centrifugation.
- ***DNA Sequencing***
- Classical chain termination sequencing uses dideoxynucleotides (ddNTPs) to terminate DNA synthesis at particular nucleotides.
- Primer - A single stranded nucleic acid molecule with a 3′ –OH used to initiate DNA polymerase replication of a paired template strand.
- ***DNA Sequencing***
- Fluorescently tagged ddNTPs and capillary gel electrophoresis allow automated, high-throughput DNA sequencing.

- The next generations of sequencing techniques aim to increase automation and decrease time and cost of sequencing.
- ***PCR and RT-PCR***
- Polymerase chain reaction (PCR) permits the exponential amplification of a desired sequence, using primers that anneal to the sequence of interest.
- ***PCR and RT-PCR***
- RT-PCR uses reverse transcriptase to convert RNA to DNA for use in a PCR reaction.
- ***PCR and RT-PCR***
- Real-time, or quantitative, PCR detects the products of PCR amplification during their synthesis, and is more sensitive and quantitative than conventional PCR.
- PCR depends on the use of thermostable DNA polymerases that can withstand multiple cycles of template denaturation.
- ***PCR and RT-PCR***
- fluorescence resonant energy transfer (FRET) – A process whereby the emission from an excited fluorophore is captured and reemitted at a longer wavelength by a nearby second fluorophore whose excitation spectrum matches the emission frequency of the first fluorophore.
- Blotting Methods
- Southern blotting involves the transfer of DNA from a gel to a membrane, followed by detection of specific sequences by hybridization with a labeled probe.
- ***Blotting Methods***
- Northern blotting is similar to Southern blotting, but involves the transfer of RNA from a gel to a membrane.
- Western blotting entails separation of proteins on a sodium dodecyl sulfate (SDS) gel, transfer to a nitrocellulose membrane, and detection of proteins of interest using antibodies.
- ***Blotting Methods***
- epitope tag – A short peptide sequence that encodes a recognition site (“epitope”) for an antibody, typically fused to a protein of interest for detection or purification by the antibody.
- ***DNA Microarrays***
- DNA microarrays comprise known DNA sequences spotted or synthesized on a small chip.
- DNA Microarrays
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- Genome-wide transcription analysis is performed using labeled cDNA from experimental samples hybridized to a microarray containing sequences from all ORFs of the organism being used.
- SNP arrays permit genome-wide genotyping of single-nucleotide polymorphisms.
- Array comparative genome hybridization (array-CGH) allows the detection of copy number changes in any DNA sequence compared between two samples.

- **LECTURE 7.** ***DNA cloning techniques***
- 3.1 Introduction
- ***Cloning vector*** **–** DNA (often derived from a plasmid or a bacteriophage genome) that can be used to propagate an incorporated DNA sequence in a host cell.
    - Vectors contain selectable markers and replication origins to allow identification and maintenance of the vector in the host.
- 3.2 Nucleases
- **Nucleases** hydrolyze an ester bond within a phosphodiester bond.
- **Phosphatases** hydrolyze the ester bond in a phosphomonoester bond.
- 3.2 Nucleases
- **endonuclease –** Nuclease that cleaves phosphoester bonds within a nucleic acid chain.
    - It may be specific for RNA or for single-stranded or double-stranded DNA.
- **exonuclease –** Nuclease that cleaves phosphoester bonds one at a time from the end of a polynucleotide chain.
    - It may be specific for either the 5′ or 3′ end of DNA or RNA.
- 3.2 Nucleases
- Restriction endonucleases can be used to cleave DNA into defined fragments.
- 3.2 Nucleases
- A map can be generated by using the overlaps between the fragments generated by different restriction enzymes.
- 3.3 Cloning
- **Cloning** a fragment of DNA requires a specially engineered **vector**.
- **recombinant DNA** – A DNA molecule that has been created by joining together two or more molecules from different sources.
- **ligating (or ligation)** – The process of joining together two DNA fragments.
- 3.3 Cloning
- **subclone –** The process of breaking a cloned fragment into smaller fragments for further cloning.
- **multiple cloning site (MCS) –** A sequence of DNA containing a series of tandem restriction endonuclease sites used in cloning vectors for creating recombinant molecules.
- 3.3 Cloning
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- **transformation** – The acquisition of new genetic material by incorporation of added exogenous, nonviral DNA.

- Blue/white selection allows the identification of bacteria that contain the vector plasmid and vector plasmids that contain an **insert**.
- 3.4 Cloning Vectors Can Be Specialized for Different Purposes
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- Cloning vectors may be bacterial plasmids, phages, **cosmids**, or **yeast artificial chromosomes**.
- **Shuttle vectors** can be propagated in more than one type of host cell.
- **Expression vectors** contain promoters that allow transcription of any cloned gene.
- 3.4 Cloning Vectors Can Be Specialized for Different Purposes
- **Reporter genes** can be used to measure promoter activity or tissue-specific expression.
- 3.4 Cloning Vectors Can Be Specialized for Different Purposes
- Numerous methods exist to introduce DNA into different target cells.
- 3.7 DNA Sequencing
- Classical chain termination sequencing uses **dideoxynucleotides** (ddNTPs) to terminate DNA synthesis at particular nucleotides.
- **Primer -** A single stranded nucleic acid molecule with a 3′ –OH used to initiate DNA polymerase replication of a paired template strand.
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