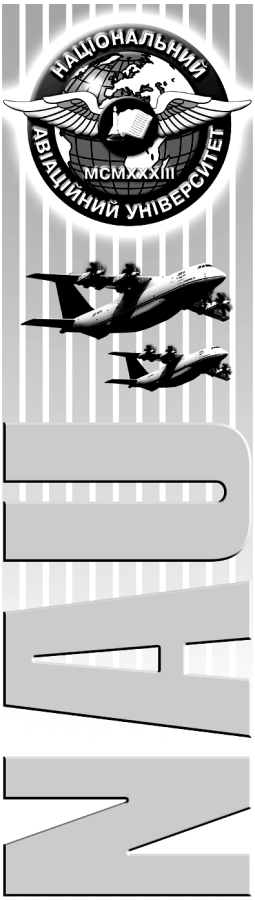

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MINISTRY OF EDUCATION AND SCIENCE OF UKRAINE
National Aviation University

MOLECULAR BIOTECHNOLOGY

Guide to Laboratory Practical Work for the students of specialties 7/8.05140103 «Pharmaceutical biotechnology»

Kyiv 2016

MINISTRY OF EDUCATION AND SCIENCE OF UKRAINE
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Guide to Laboratory Practical Work
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«Pharmaceutical biotechnology»

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Reviewer *I. V. Matvieieva*

Approved by the Methodical and Editorial Board of the National Aviation University (Minutes # 4/16 of 15.04.2016).

У практикумі наведено методики виконання лабораторних робіт з курсу «Молекулярна біотехнологія», коротке теоретичне обґрунтування кожного досліду, а також контрольні запитання з теоретичної та практичної частин.

Для студентів спеціальностей 7/8.05140103 «Фармацевтична біотехнологія»

М79 **Molecular biotechnology** : Guide to Laboratory Practical Work / compilers : S. I. Tarasyuk, O. A. Vasylchenko, Yu. M. Hlushko, L.V. Kucheryava. – K. : NAU, 2016. – 44 p.

The methods for performing laboratory works on «Molecular biotechnology» and short theoretical substantiation of each experiment are represented. The guide contains test questions on the theoretical and practical parts.

For the students of specialties 7/8.05140103 «Pharmaceutical biotechnology».

INTRODUCTION

The main purpose of the Guide to Laboratory Practical Work "Molecular biotechnology" is consolidating and expanding students' knowledge, improvement of practical skills in the laboratory, mastering modern methods of molecular biotechnology, research with application of biological objects for possible further implementation in biotechnological production or use in scientific work.

The Guide contains guidelines for students' laboratory works performing. During the implementation of proposed experiments students improve knowledge of experimental research molecular biotechnology methods, acquire the ability to analyze obtained results.

A special section is dedicated to the safety rules during the work in the laboratory.

The Guide contains material according to two modules of the discipline. The first module is "Basics of molecular biotechnology"; the second module is "Nanobiotechnology". Execution of laboratory works gives the possibility to master modern methods of molecular biotechnology.

PRECAUTIONS TO LABORATORY WORKS

1. In biochemical laboratories should be available the fire-prevention equipment (fire extinguisher, sand, water), first aid kit and personal protection means (rubber gloves, goggles, etc.).

2. In the laboratory it is forbidden to eat, smoke.

3. It is necessary to work in special clothes (coats).

While working with large volumes of concentrated acids and their pouring, extra protection goggles should be used.

4. After working with reagents it is necessary to wash hands, after working with toxic compounds – wash hands, brush your teeth, and wash your mouth, change clothes.

5. All reagents must be in good condition according to the storage requirements.

6. Poisonous and explosive substances should be stored in the safe.

7. While working with reagents do not touch the face, eyes and open areas of skin.

8. Works relating to the release of gases have to be done only under the air hood.

9. Do not taste reagents, do not inhale volatile substances – they may be poisonous.

10. Solutions should be only picked up with the pipettes, using a special harness with rubber bulb or dozer.

12. Before investigations it is necessary to carefully examine the technique and prepare the work place.

13. Before using the device one should read the instructions.

14. Follow safety rules while working with electrical appliance and equipment.

15. If acids or alkalis get on your skin, affected areas should be washed immediately with plenty of water (under the water jet).

Module I

BASICS OF MOLECULAR BIOTECHNOLOGY

Laboratory work 1

MICROMETHOD OF PERIPHERAL BLOOD LYMPHOCYTES CULTURING FOR CHROMOSOMAL ANALYSIS

Aim of work: to obtain the culture of human peripheral blood cells, to prepare metaphase plates for chromosomal analysis.

Main tasks of work

1. Prepare sterile nutrient medium for culturing human peripheral blood lymphocytes.
2. Gain skills of cells cultivation under sterile conditions.
3. Make chromosomal preparations and carry out their staining.
4. Investigate the human karyotype with the microscope and make the karyogram.

Basic theoretical information

Cytogenetic analysis (method of chromosome analysis) is based on the study of the structure and number of chromosomes with microscopy, which allows recording a diagnosis of hereditary diseases in the form of karyotype formula.

Karyotype is a specific for each type of organisms set of chromosomes, which is characterized by a certain number of chromosomes and peculiarities of their structure. The appearance of chromosomes changes significantly during the cell cycle: in the interphase chromosomes are despiralized (filamentous) and localized in the nucleus.

For karyotyping the cells in one of the stages of their division – **metaphase of mitosis** are used, as at this stage of cell division chromosomes are as much as possible spiralized, shortened, thickened and are in the equatorial zone of the cell. To determine the human karyotype whether mononuclear cells isolated from blood samples, the division of which is stimulated by addition of mitogens, or cells cultures normally actively dividing (fibroblasts of the skin, bone marrow cells) are usually used. Enrichment of the cell culture population is carried out with the stoppage of cells division in metaphase of mitosis with addition

of **colchicine** – alkaloid blocking the formation of spindle microtubules and chromosomes disjunction to the poles, thus preventing the completion of mitosis.

In order to systematize cytogenetic descriptions International System for Cytogenetic Nomenclature (ISCN) was developed based on the differential staining of chromosomes, which allows describing in detail individual chromosomes and their regions. The record is as follows:

[*Quantity of chromosomes*], [*sex chromosome*], [*number of chromosomes*], [*arm*], [*region number*], [*band number*]. For example, human karyotype is written as follows: 46, XX (woman) and 46, XY (man) (Fig. 1).

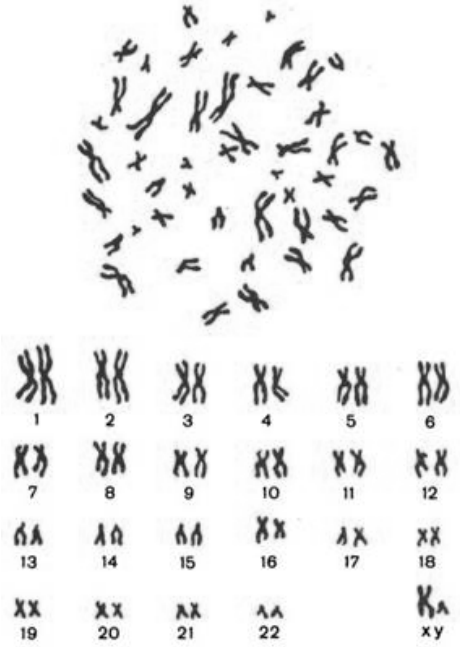


Fig. 1. Man normal karyotype

A long arm of chromosome is represented by letter **q**, a short one – by the letter **p**, **FN**– fundamental number, the total number of chromosomal arms; **2n**– diploid chromosome set.

Disruptions of the normal karyotype of the human occur at early stages of the organism development and are usually accompanied by multiple malformations. Most of these anomalies are fatal. It is

established that chromosomal abnormalities cause half of miscarriages and 5–7 %of stillbirths. However, quite a big number of children (about 6 %) have congenital and hereditary diseases.

Equipment, tools and materials

Box with bactericidal lamp; refrigerator; thermostat at 37 °C; centrifuge at 3 000 rpm; scarifier (blood lancet); micropipettes; 1, 10 and 20 ml graduated pipettes; hemocapillary; sterile penicillin vials with stoppers fixed with plaster; centrifuge tubes; slides; Hanks nutrient medium(medium № 199); phytohemagglutinin (PHA); 0.02 % colchicine solution in saline; hypotonic solution (0.075 M KCl solution or 0.44 % trisubstituted sodium citrate solution); ethanol and acetic acid (3:1) mixture; whole inactivated serum of cattle; heparin; dyes (azure, eosin and silver nitrate).

Work procedure

I. Cultivation of human peripheral blood lymphocytes

Disinfect, degrease and dry a little the skin of the finger. It should be wiped with sterile cotton wool soaked in a mixture of alcohol and ether. Squeeze the phalanx pulp on each side and with rapid movement of the scarifier pierce the skin at a depth of the blade, so that blood comes out without pressing.

When a blood drop appears, it is necessary to put under it a sterile hemocapillary holding it horizontally and gently (not to close the hole) pressing it to the skin. Blood fills the capillary tube (according to the law of capillarity). Collect quickly blood to the mark (0.5 ml). After collecting blood to the puncture put a cotton ball soaked in 5 % iodine alcohol solution and press it by the thumb of the same hand.

Put blood into a sterile penicillin vial containing 0.1 ml of 20 times diluted heparin, and close with a sterile rubber stopper, in which was additionally introduced a needle for access of air. Blood prepared in this way can be stored in the refrigerator up to three days.

In a vial with phytohemagglutinin (PHA –refined myogen extracted from beans) introduce 5 ml of sterile bidistilled water or 0.9 % NaCl solution. With a sterile micropipette take 0.015 ml of PHA and enter into the vial with blood. Afterwards, with a sterile pipette (2 ml) enter 1–2 ml of Hanks medium (or medium № 199) and 0.5 ml of inactivated cattle serum. Close the vial

hermetically with a sterile stopper, shake gently and place in the thermostat at 37 °C for 72 hours. The vials should be shaken every day at the same time.

II. Preparation of metaphase plates

In the cell culture with PHA lymphocytes enter daily into a mitotic cycle. After 54–72 hours the maximum number of mitoses is gained. In non-sterile conditions into the vial with the culture introduce 0.1 ml of colchicine (1 mg per 100 ml of 0.9 % NaCI solution). Added to the culture colchicine stops mitosis at metaphase stage.

After 2 hours the content of the vial transfer into centrifuge tubes, treat cells with hypotonic solution (add 5 ml of heated to 37 °C 0.55 % potassium chloride solution for 5–8 min). Carry out centrifugation for 8 minutes at 1000 rpm. With a pipette remove the supernatant, and layer slowly on the precipitate 3–4 ml of alcohol and acetic acid mixture (3:1).

After 30 minutes the tube content mix thoroughly and centrifuge for 8 minutes at 1 000 rpm; and remove some portion of the supernatant. If the supernatant is reddish black, you should repeat fixation two or three times. On the degreased slides, washed with a chromium mixture, which are stored in a refrigerator in 10 % water solution of ethanol, put the cells suspension using a crushed drop method from 30 cm height. Put 3–4 drops on each slide. The slides should be dried a little in a thermostat at 37 °C.

III. Staining of chromosome preparations

To obtain a classical karyotype different staining techniques of chromosomes using dyes or their mixtures are used. Depending on binding of a dye with different parts of chromosomes, staining is uneven and characteristic striped structure (the complex of cross marks, eng. *Banding*) is formed, reflecting the linear heterogeneity of the chromosome that is specific to the homologous pairs of chromosomes. Various complexes of dyes are used. These techniques are generally referred to as differential staining of chromosomes:

– **G-staining**– modified staining by Romanovsky – Gimsa. Used as a standard method of cytogenetic analysis. It is used in small aberrations and marker chromosomes detecting;

– **C-staining**–is used for the analysis of centromeric chromosome regions containing constitutive heterochromatin and variable regions of Y-chromosome;

– **T-staining**–is used for the analysis of telomeric chromosomal regions;

– **Q-staining**–staining by Kasperson with acrichin-mustard with further observation under a fluorescent microscope. The study of Y-chromosome (quicker determination of genetic sex, detection of translocations between X- and Y-chromosome or between Y-chromosome and autosomes) is mostly used;

– **R-staining**–acridine orange and similar dyes are used; in this case chromosomes regions insensitive to G-staining are stained.

With a microdispenser put on metaphase chromosomes preparations 50 ml of bidistilled water, 150 μl of 50 % silver nitrate ($AgNO_3$) solution and 100 ml of 2 % gelatin solution. Mix well, put a cover glass and place in the thermostat for 8–10 minutes at a temperature of 56 °C. After 10 minutes wash off the dye, dehydrate preparations with 70 % ethanol and let the smear dry up in the thermostat for 10 minutes. Stain again the smears with Romanovsky-Gimsa dye (dip slides in the glass with dye solution for 15–20 min). Rinse the smears using running water, dry in the air. Stained preparations are microscoped with immersion oil with an increase of 10 × 100.

Processing of experimental data

To obtain objective results it is necessary to analyze about 20 or more metaphase plates, the best of which it is necessary to photograph. Cut chromosomes of printed metaphase plates photos and make karyograms.

Analysis of results

Basing on the obtained results, draw conclusions on the state of the karyotype and the level of chromosome aberrations.

Test questions

1. What is karyotype?
2. How to write an individual karyotype according to the international nomenclature?
3. What chromosome aberrations and genome mutations can appear and how are they classified?
4. What is the mechanism of appearance of Robertson's translocations?
5. What is the aim of defining the karyotype disorders?

Sources: [1].

Laboratory work 2

MOLECULAR AND CYTOGENETIC METHOD OF FLUORESCENT *IN SITU* HYBRIDIZATION (FISH) IN DIAGNOSIS OF COMPLEX FORMS OF CHROMOSOMAL DISORDERS

Aim of work –to perform a fluorescent *in situ* hybridization (FISH) in diagnosis of complex forms of an individual's chromosomal aberrations.

Main tasks of work

1. To get skills in preparing cytogenetic preparations.
2. To master the technique of fluorescent DNA samples preparing.
3. To get skills of working with a fluorescent microscope.
4. To assess the quality of cytogenetic preparations.

Basic theoretical information

In cases of complex chromosomal rearrangements, hidden deletions in a clinical picture of a particular syndrome associated with microstructural abnormalities of chromosomes, it is necessary to additionally analyze the karyotype involving molecular and cytogenetic methods of fluorescent *in situ* hybridization (FISH). FISH-diagnosis consists in staining chromosomes with a set of fluorescent dyes binding to specific regions of chromosomes (Fig. 2).

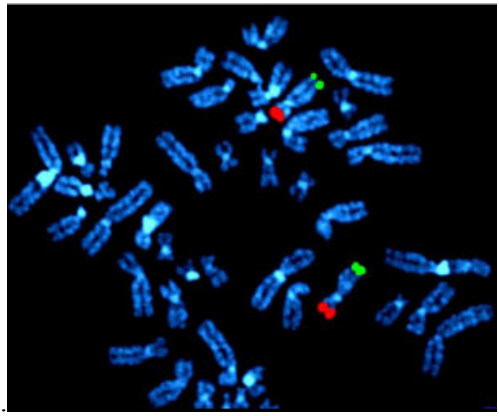


Fig. 2. Individual's metaphase plate stained with fluorescent dyes

As a result of this staining homologous pairs of chromosomes receive identical spectral characteristics, which not only facilitates identification of such pairs, but also facilitates the detection of interchromosomal translocations, that is the exchange of nonhomologous regions between non-homologous chromosomes, as translocated regions have a spectrum different from the spectrum of the remaining chromosomes.

Molecular and cytogenetic diagnostics (pre- and postnatal) of chromosomal abnormalities by FISH are carried for probands with multiple congenital developmental disorders caused by certain chromosomal aberrations, however not identified or corrected by standard methods of chromosomes staining, for example, in families with reproductive disorders, patients with clinically assumed microdeletion syndromes (syndrome of Angelman, Williams, Wolf-Hirschhorn, Di Giorge/ del22q11.2, Miller-Dieker, Prader-Willi, Smith-Magenis etc). To carry out FISH on cytological preparation it is necessary to experimentally denature DNA, afterwards put a labeled DNA on the preparation and carry out renaturation. A labeled DNA will replace the native chain in that place of the chromosome where there is a homologous DNA fragment. Using this technique it is quite easy to define gene localization or DNA fragment in the chromosome. To do this you need to have labeled DNA fragments – probes.

Equipment, tools and materials

Container with a bactericidal lamp; refrigerator; thermostat at 37 $^{\circ}$C; centrifuge at 3 000 rpm with the vibration mixer (vortex) function; hybridization oven (hermetically closed vial with the capacity not exceeding 0.5 dm^3, able to hold a given temperature and high humidity during 24 hours); dry-air sterilizer, light microscope with a fluorescent module.

Work procedure

I. Preparing of cytogenetic preparations for FISH

For FISH cytogenetic preparations of peripheral blood lymphocytes, fibroblasts, buccal epithelium cells, chorionic villi or placenta are used. Sampling of biological material and cell culture is carried out using standard techniques. The hybridization *in situ* of the molecular DNA-probe on the target chromosome is carried out on a particular limited area of the slide. The cytogenetic preparation area must contain a sufficient quantity of the cellular material and a sufficient number of

metaphase plates. The suspension of cells for application on the slides should have a bigger concentration of cell material if compared to the concentration used for making preparations for staining using a GTG-method. The optimal dilution of the suspension is achieved empirically. Preparations for FISH are prepared 3 days before the hybridization and stored at room temperature.

In order to obtain high quality preparations it is necessary to carry out a preliminary enzymatic treatment of preparations:

1. Heat 100 ml of 10 mM HCl solution up to 37 °C and add 0.5 ml of the pepsin work solution.

2. Incubate the preparation in the pepsin work solution of at 37 °C for 5 minutes, and then wash in the buffer PBS №1 at room temperature for 5 minutes.

3. Incubate the preparation in the solution of formaldehyde at room temperature for 10 min (100 ml of solution put on the area of hybridization and cover with a cover slide). Wash the preparation in the buffer PBS №2 at room temperature for 5 min and fix in alcohols of different concentrations (70, 85 and 96 %) for 3 minutes in each.

II. Preparing of fluorescent DNA-probes and FISH carrying out

Working with fluorescent components of reagents it is necessary to avoid penetration of the direct light. For the purposes of diagnostic they use: LSI – locus-specific molecular DNA-probes, WCP–whole chromosome DNA-probes and other types of oligonucleotides.

1. Defrost at room temperature and carefully resuspend components of the hybridization solution (probe and transfer buffer): centrifuge in the microcentrifuge for 1–3 sec, mix in the vibration mixer (vortex). Repeat the step twice.

2. Mix in the microtube of eppendorf type the components of hybridization mixture (hybridization buffer, DNA-probe, distilled water) in proportions according to the recommendations of the manufacturer. The size of the hybridization zone in the cytogenetic preparation has a clear dependence on the amount of hybridization mixture (24×50 mm^2 – 10 ml, 18×18mm^2 – 5 ml). The resulting hybridization mixture mix thoroughly in the vibration mixer (vortex) and centrifuge in the micro centrifuge for 1–3 sec.

3. To perform the codenaturation and hybridization it necessary to prepare beforehand and put in the thermostat a hybridization chamber. Drying up the chamber is not allowed during hybridization. On the walls of the chamber put a layer of filter paper and moisten generously with

distilled water, fill the bottom with distilled water up to 0.5 cm layer, put a tripod to hold the preparation on the slide in the horizontal position.

4. Heat the electric hot plate up to the required temperature specified in Table. 1, apply the required amount of the probe on the preparation hybridization zone and cover immediately with a piece of cover slide (formation of air bubbles is inadmissible).

5. Glue up the edges of the cover slide with rubber adhesive without leaving gaps. Place the preparation in the thermostat and perform codenaturation according to the conditions specified in the Table 1. Afterwards, put the preparation horizontally in the prepared hybridization chamber and place in the thermostat during hybridization (Table 1).

Table 1

Conditions for performing FISH for main types of DNA-probes

Probe	Denaturation temperature, ^{o}C	Denaturation time, min	Hybridization temperature, ^{o}C	Hybridization time, h
WCP	68–75	5	37	18
LSI	73–75	3–5	37	18

6. Wash off the preparation after hybridization. In order to obtain clear quality fluorescent signals on the target DNA it is necessary to avoid direct light on the preparation; to control the temperature and volume of washing solution. In three beakers pour separately solutions for washing: №1 – 70 ml 0/4 × SSC / 0.3 % NP-40; №2 – 2 × SSC / 0.1 % NP-40; №3 – H_2O_{dist}.

a) 0.4 × SSC / 0.3 % NP-40 pH 7.0–7.5 (correct pH by 1M NaOH), 20 ml of 20 × SSC; 950 ml of H_2O; 3 ml of NP-40. Mix and fill the volume up to 1 000 ml with H_2O_{dist}.

b) 2 × SSC / 0.1 % NP-40 pH 7.0 ± 0.2 (correct pH by 1M NaOH): 950 ml of 2 ×SSC; 1 ml of NP-40. Mix and fill the volume with H_2O to 1 000 ml.

c) 2 × SSC pH 7.0 ± 0.2 (bring the pH 1M NaOH): 100 ml of 20 × SSC; 850 ml of H_2O. Dissolve and fill the volume with H_2O to 1 000 ml.

d) 20 × SSC pH 5.3 (correct pH using 1M NCl): 175.3 g of NaCl; 88.2 g of $Na_3C_6H_5O_7 \times 2\ H_2O$; 800 ml of H_2O.

Dissolve salts and fill the volume up to 1000 ml with H_4O). In a water bath heat the first solution up to the temperature of 73 ± 1 °C, the

other ones should be of room temperature (solutions for washing should be used during the day).

7. Separate the cover slides from solution №1, wash off the preparation for 2 min, shaking every 3 sec. Transfer the preparation into the beaker with solution №2 and wash for 1 min. Then wash in distilled water for 5 seconds.

8. Treat the preparation with 70, 85 and 96 % alcohols sequentially for 1 minute with each. Dry the preparations in the air to a complete evaporation of alcohol.

9. Put 10 µl of basic dye on the region of hybridization and cover with a cover slide. For fluorochromes *green* or *aqua* PI or DAPI light filters are used, for orange – only DAPI. Store the preparations in the darkness at t° – 20 °C.

Processing of experimental data

The quality of cytogenetic preparations must be assessed using a fluorescent microscope with a set of appropriate filters and magnification factor × 200 (Fig. 3).

Fig. 3. Light microscope Primo Star with a fluorescent module iLED

Requirements to the preparation are high density of the material on the glass, isolation and integrity of metaphase plates. The analysis of signals on metaphase chromosomes includes examination of at least 20–25 metaphases with absence of mosaicism. To clarify the status of the mosaic karyotype 100 metaphases are examined. In the experiment they take into account metaphases, in which on both analyzed homologous chromosomes the control fluorescent signals in the composition of the DNA-probes are identified. The analysis of signals on interphase chromosomes includes examination of at least 50 interphase cells.

Analysis of results

The cytogenetic analysis concludes with record-making of results and inputting photos into a computer database. A patient's karyotype, studied using molecular cytogenetic method FISH, is recorded in accordance with the rules of the International system of nomenclature of human chromosomes (ISCN), indicating the type of DNA-probe used.

For example, 47, XY, + mar.ish dup (18) (p10) (wcp18 +). The record means that the resulting standard cytogenetic analysis in a male karyotype revealed 47 chromosomes, and an additional marker chromosome was also identified. The record of this information is limited with a full stop. Then follows, without space, the abbreviation "ish", which means «in situ hybridization», and after space the results of molecular cytogenetic studies are recorded. A marker chromosome is that one formed by inverted duplication of short arm of chromosome 18 around the centromere point 18p10. After completion of the analysis the conclusion is drawn about the results of molecular cytogenetic diagnostics (Appendix 1).

Test questions

1. Explain when molecular and cytogenetic diagnosis of chromosomal disorders is used?
2. How is a human karyotype recorded according to the international nomenclature?
3. What DNA-probes can be used for FISH?
4. What is the principle of fluorescent DNA-probes preparing and FISH performing?

Sources: [2]; [3].

Laboratory work 3

ISOLATION OF GENOMIC DNA FROM PLANT MATERIAL

Aim of work –to master modern methods of high quality plants genomic DNA isolation.

Main tasks of work

1. To prepare homogenates of plant material.
2. To gain skills of plants genomic DNA isolation.

Basic theoretical information

In the process of transformation of plants the transformation and integration of foreign genes into plant cells is done. To identify a successful carrying out of transformation the molecular genetic studies of plants regenerants and their offspring are performed. Transgenic organisms can be quickly identified using a small amount of DNA based on PCR (polymerase chain reaction), but successful screening of a large number of amplified fragments during PCR requires the use of appropriate methods for the isolation of DNA.

Methods of high-quality DNA isolation for PCR require a small amount of plant material, but a high degree of purification of genomic DNA.

A genomic DNA is isolated from chromatin of chromosomes (Fig. 4), which in turn represents a set of DNA, RNA and histone and non-histone proteins.

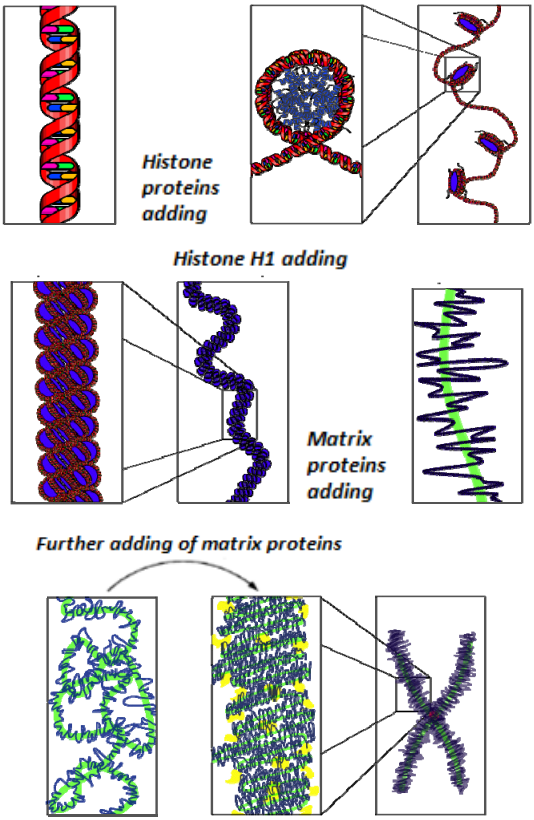


Fig. 4. Chromosome structure

Equipment, tools and materials

Thermostat, ultracentrifuge, centrifuges with Vortex, microdispensers, microtubes of eppendorf type, porcelain mortar with pestle, sterile quartz sand, glass rods. The list of reagents required to perform the work is listed in Table. 2.

Table 2

Buffers and solutions for DNA extraction

Buffer for extraction A (EBA):	**for 100 ml**
2 % hexadecyltrimethylammonium bromide (CTAB)	2.0 g
100 mM tris (pH 8.0)	10 ml
20 mM EDTA (use 0.5M bittern)	1 ml
1.4 M NaCl	8.2 g
0,1 % ascorbic acid	0,1 g
10 mM β-mercaptoethanol (BME) (use14.3 M bittern)	70 µl
Buffer for extraction B(EBB):	**for 100 ml**
100 mM tris-HCl (pH 8.0) (use 1M bittern)	10 ml
50 mM EDTA (use 0.5 M bittern)	2.5 ml
100 mM NaCl	0.6 g
10 mM β-mercaptoethanol (BME) (use14.3 M bittern)	70 µl
Tris EDTA (TE) buffer:	**for 100 ml**
10 mM tris (pH 8.0) (use 1M bittern)	1.0 ml
1mM EDTA (0.5 M concentrating solution)	50 µl
Other necessary reagents:	
20 % sodium dodecyl sulphate (SDS); 5 M potassium acetate or5 M sodium chloride (keep at –20 °C); 3 M sodium acetate (pH 5.2); 70 % C_2H_5OH (keep at – 20 °C); Absolute isopropanol (keep at – 20 °C); Chloroform	

Work procedure

1. Weigh 3 g of plant material (fresh or frozen fruit), transfer the tissue to a clean porcelain mortar with pestle, add sterile sand and grind the plant material until the smooth (prepare homogenates).

2. Transfer quickly the tissue into microcentrifuge eppendorfs of 1.5 ml.

3. To each specimen 300 µl of buffer for extraction A (EBA), 400 µl of buffer for extraction B (EBB) and200 µl of 20 % sodium dodecyl

sulfate (SDS) should be added. Then hold the tubes shaking in the centrifuge (vortex) during 10 min.

4. Carry the tube with studied material onto a test-tube rack and put it in the thermostat for 10 minutes at 65 ^{o}C. Next, it is necessary to put the tubes on ice and add 410 ml of cold potassium acetate to precipitate the proteins and then mix the suspension, turning the microtubes several times and place eppendorfs on ice for 5 min.

5. For the precipitation of proteins the centrifugation is performed at 13 000 rpm during 15 minutes. (If possible, this step is performed at 4 °C). Next, it is necessary to add to each tube 410 ml of chloroform and to centrifuge for 10 minutesat 3000 rpm.

6. If the researcher meets all the conditions, the supernatant must contain the DNA. With the microdispenser it is necessary to transfer1 ml of the supernatant to a new microcentrifuge tube of 1.5 ml, add 540 ml of ice cold absolute isopropanol and put on ice for 20 minutes. For the precipitation of DNA molecules it is necessary to perform centrifugation at 10000 rpm during 10 min. Then pour out carefully the supernatant, wash the residue in 200 ml of 70 % ethanol and leave it to dry for 10 minutes, having turned over the tube on the filter paper. The precipitated DNA should be dissolved in 20 ml of TE buffer and kept in the freezer for further research.

Processing of experimental data

The quality of plant DNA isolation is assessed by electrophoretic separation of DNA fragments with ethidium bromide in agarose gel performing and the results visualizing with a transilluminator.

Analysis of results

Depending on the luminescence intensity draw a conclusion on the quality of genomic plant DNA isolation.

Test questions

1. Explain how are plant DNA used in molecular biotechnology?
2. What plant material and why can be used for DNA isolation?
3. List the main criteria for assessing the quality of the resulting DNA?

Sources: [4].

Laboratory work 4

GENOMIC DNA ISOLATION FROM PERIPHERAL BLOOD LYMPHOCYTES OF MAMMALS

Aim of work – to master modern ways of isolation of quality genomic DNA of mammals

Main tasks of work

1. To learn basic methods of mammals genomic DNA isolation.
2. To gain skills of mammals genomic DNA isolation.

Basic theoretical information

DNA isolation procedure has some features that need attention to be paid to when performing work. So, after a phase of the cell membrane destruction and lysis the cell homogenate has to clear up and become viscous. The higher the viscosity of the solution is, the higher is the probability of receiving high-polymeric fragments of the cellular DNA. Deproteinization of the preparation by organic agents should not significantly affect the viscosity of the mixture containing a sufficient concentration of DNA molecule with high molecular weight. If, after clearing the sample by organic agents, there is a sharp reduction or complete viscosity absence, then one of possible causes is considerable fragmentation of DNA macromolecules.

Equipment, tools and materials

Thermostat, ultracentrifuge, microdispensers, microtubes of eppendorf type, porcelain mortar with pestle, sterile quartz sand, glass sticks, heparinized animal blood, lytic buffer№ 1 (75 mM NaCl, 25 mM EDTA) and buffer№ 2 (10 mM NaCl, 10 mM EDTA), 20 % solution of sodium sarcosyl, 7.5 M ammonium acetate solution, 6 M solution of guanidine hydrochloride, 10 mg / ml of proteinase K solution, cold (–20 °C) 96 and 70 % ethanol, TE buffer, paraffin film (Parafilm® M), 100 ml conical flasks, centrifuge with cooling, 30 ml centrifuge tubes, ice bath, thermostat or water bath (temperature 50–60 °C), refrigerator, sampler and tips for it, eppendorfs, marker, glass rods.

Work procedure

4.1. Washing off and concentrating of lymphocytes

1. Mix thoroughly heparinized blood (1 ml) with 20 ml of buffer № 1. Incubate on ice for 15 minutes.
2. Precipitate leukocytes by centrifugation at 6 000 rpm at 4 °C for 10 min.
3. Pour out the supernatant containing lysed red blood cells.
4. Resuspend the precipitate by a small amount of buffer № 2.
5. Balance the test sample by the similar tube with water.
6. Centrifuge at 6 000 rpm at 4 °C for 10 min.
7. Pour out the supernatant. If the precipitate is colored, repeat washing off by buffer №2 (p. 4) until the color disappears.

4.2. Lymphocytes lysis

1. Add 350 ml of 20 % sodium sarcosyl solution.
2. Add 250 ml of 7.5 M ammonium acetate solution.
3. Add 3.5 ml of 6 M guanidine hydrochloride solution.
4. Add 125 ml of 10 mg / ml proteinase K solution (to a final concentration of 300 μg/ ml).
5. Incubate the sample at 50–60 °C for 2 hours.
6. Transfer the sample on ice and cool to 0 °C.

4.3. Precipitation of the DNA with ethanol

1. Precipitate the DNA adding 10 ml of cold 96 % ethanol.
2. Rinse the received "jellyfish" with a portion of 70 % ethanol.
3. Centrifuge the DNA for 5 min at 4 °C at 4–10 000 rpm or wind it around the glass rod.
4. Transfer the rod with the DNA in the eppendorf with 70 % ethanol and seal it hermetically with paraffin film. Store the received preparation at a temperature of –20 °C.
5. Pour out alcohol from DNA preparation. Dry the DNA precipitate and dissolve it in the TE buffer. Keep at temperature of –20 °C.

Processing of experimental data

Typically, while precipitated with alcohol, these DNAs form the precipitate which cannot be winded around the glass rod. Fragments of the DNA clinging to the glass rod have a length of about 20 thousand

pairs of nucleotides and more. Generally, the yield of the DNA from leukocytes is 30–60 μg / ml of blood.

Analysis of results

Basing on your own observations and the results received draw conclusions on the peculiarities of the process of chromosomal DNA preparations isolation from lymphocytes.

Test questions

1. Explain basic methodological differences of plant and mammals DNA isolation.
2. What biological material can be used to extract a high-quality mammals DNA?
3. List the main criteria for assessing the quality of the resulting DNA.

Sources: [5].

Laboratory work 5

TOTAL EUKARYOTIC RNA OBTAINING

Aim of work –to master the methodology of total eukaryotic RNA preparations isolation.

Main tasks of work

1. To study the main methods of the RNA isolation.
2. To isolate the RNA from plant material.

Basic theoretical information

For obtaining strains of gene engineering origin it is necessary to clone a target gene. An alternative to "cutting" the gene using restrictase of native genomic DNA is its enzymatic synthesis on the basis of the messenger RNA (mRNA). This became possible only after 1972 when H. Temin and D. Baltimore (USA), studying retroviruses, discovered a reverse transcriptase or revertase – an enzyme performing DNA synthesis from the RNA matrix. The revertase is widely used in molecular cloning.

The general plan of action to obtain target genes by this method consists in the following. Firstly, from eukaryote cells it is obtained the total RNA preparation from which an individual mRNA, corresponding to a certain protein, is isolated. The reverse transcriptase catalyzes the

synthesis of the first DNA strand, complementary to mRNA. The result is the RNA-DNA hybrid. Then, matrix RNA is removed by alkaline hydrolysis method or by enzyme RNAase. The resulting single-stranded DNA is called complementary DNA or cDNA and is used as a matrix for the synthesis of the second DNA strand that is synthesized by DNA polymerase I (it works only on DNA matrix) or revertase. In the latter case the loop connecting the first and second strands of cDNA, is split by nuclease (enzyme that specifically destroys the single-stranded DNA segments). The complete DNA copy of the mRNA contains all the information for protein synthesis, which corresponds to the structural part of a certain gene without introns and other nontranscribed sequences proper to the most of eukaryotic genes. The resulting gene is included into the vector, using which the desired genetic information is transferred to a producer cell, where the synthesis takes place.

The content of RNA in a typical mammalian cell is about 10^5μg.The RNA is a nucleic acid different from the DNA by functions, chemical composition and secondary structure. Depending on the functions there are three major classes of RNA:

1. Ribosomal RNAs (rRNA) make up the ribosome (usually 285, 185 and 55). In total cellular RNA pool their share is 80–85 %.

2. Transfer RNAs (tRNA) transfer amino acids to the place of protein synthesis. Low molecular weight RNAs (nuclear, tRNA, etc.) make up 10–15 % of RNA total amount.

3. Messenger RNAs (mRNA) are molecules formed during the transcription process, that is "reading" information from specific genes, and are the template for protein synthesis. The content of this type of RNA is 1–5 % of RNAs total amount.

Equipment, tools and materials

Wheat (corn, peas, etc.) germs or plants leaves from upper layers, liquid nitrogen, STTEN buffer (6 % sucrose, 40 mM tris-HCl with pH 8.4 % triton X-100, 25 mM EDTA, 1 M NaC1), diethyl ether, mixture of chloroform with isoamyl alcohol (24:1), 6 M HCl, TE buffer, cold (– 20 °C) 70 % ethanol, rubber gloves, mortar for grinding a tissue, scissors for crushing plant material, tweezers, filter paper, Petri dish, scalpel, glass rods, glass for washing the sample, scales, eppendorfs, marker, conical flask of 100 ml, centrifuge providing 8–10 000 rpm, centrifuge tubes of 20–30 ml, ice bath.

Work procedure

I. The destruction of the cell wall

1. Weigh 0.5–1 g of leaves. Rinse with distilled water in the glass at a temperature of 2 °C. All subsequent manipulations should be carried out in ice bath conditions.

2. Crumble the leaves with scissors or razor. This can be done in the Petri dish. Move the pieces of plant tissue in the cooled mortar.

3. Add a small amount of liquid nitrogen and quickly freeze the sample.

4. Grind carefully the frozen tissue to fine-dispersed state, preventing its defrosting. To do this, pour periodically the liquid nitrogen in small portions into the mortar. In the absence of liquid nitrogen, it is recommended to pour the diethyl ether over tissue pieces and let it stand for 2 minutes with constant stirring. Then, remove ether, rinse the sample with cold distilled water and homogenize in the cooled to 0–4 °C corresponding buffer.

II. NA extraction and deproteinization

1. Using a scalpel carry quickly the received powder into the prepared in advance flask containing 5 ml of STTEN buffer and 5 ml of chloroform. Suspend thoroughly the mixture, preventing creaming of organic and aqueous phases for 15 minutes.

2. Transfer the suspension into centrifuge tubes and balance them. Centrifuge at 8–10 000 rpm for 20 min at 4 °C.

3. Transfer carefully the aqueous (upper) phase into clean cones (or tubes) so that not to spot it with protein sediment from interphase. Assess the volume of the selected phase. The organic phase with denatured protein and cellular debris pour out into a special dish for organic waste reagents.

4. Add to the selected aqueous phase the same volume of chloroform and repeat extraction with this organic agent according to paragraphs 2–3 to the complete extinction of the protein interphase**.**

III. RNA separation from DNA molecules

1. Measure the amount of the obtained aqueous phase and add 6M HCl in the amount of ½ of this volume to a final concentration of 3 M.

2. Incubate on ice not less than 1 hour. At this stage the specimen is kept according to these conditions overnight for more complete separation of RNA and DNA molecules. At the same time the DNA remains in the solution, and the RNA precipitates.

3. Centrifuge the specimen at 8–10 000 rpm. for 20 min at 4 °C.

IV. Precipitation of the RNA with alcohol

1. Dry the resulting precipitate of the RNA in the air and dissolve in TE buffer or water.

2. Add 2 volumes of the cold ethanol (–20 °C) or 0.7 of volume of the isopropanol. The optimal time required for precipitation depends on the amount of the RNA ranging from 2 to 12 – 16 hours at a temperature of –20 °C. If in alcohol during NA precipitation precipitates salt, and the solution becomes non-transparent, add 70 % ethanol or 50 % isopropanol into distilled water until the solution becomes transparent.

3. Prevent the precipitation of salt in alcohol by reducing its concentration in the sample. For this purpose the concentrated NA preparation dilute 2–3-fold with water and only after that add the appropriate amount of ethanol or isopropanol.

4. The RNA preparation is precipitated from the alcohol mixture with centrifuging at 8–10 000rpm for 20–30 min at 4 °C.

5. The RNA precipitate is treated sequentially with cold 70 % ethanol, 0.1 M sodium acetate solution, 3 M sodium acetate solution with further centrifugation at 8–10 000 rpm at 4 °C for 20–30 minutes. Then the RNA preparation is washed again with 70 % ethanol and the precipitate is gently dried and dissolved in sterile water or buffer for further work.

V. Preparing of the RNA preparation for use

1. The RNA preparation is precipitated from the alcohol mixture with centrifugation at 8–10 000 rpm for 20 – 30 min at 4 °C.

2. The RNA precipitate is treated sequentially with cold 70 % ethanol, 0.1 M sodium acetate solution, 3 M sodium acetate solution with further centrifugation at 8–10 000 rpm at 4 °C for 20–30 minutes. Then the RNA preparation is washed again with 70 % ethanol and the precipitate is gently dried and dissolved in sterile water or buffer for further work.

Processing of experimental data

Characterize the obtained RNA total preparations and draw conclusions basing on observations of the process of RNA isolation.

Analysis of results

Assess the quality of isolated RNA preparations. High quality RNA total preparations isolated from eukaryotic cells, when precipitated with alcohol, form a fine-dispersed white precipitate.

Test questions

1. Explain what different classes of nucleic acids exist.
2. Explain what use mRNA shave in molecular biotechnology.
3. What basic methods of the RNA isolation do you know?
4. What are the peculiarities of total plants RNA isolation?

Sources: [5, 6].

Module II

NANOBIOTECHNOLOGY

Laboratory work 6

POLYACRYLAMIDE GEL ELECTROPHORESIS OF PROTEINS (PAAG)

Aim of work– to master the technique of electrophoretic separation of mammals plasma proteins.

Main tasks of work

1. To study the sectors using the results of electrophoretic separation of proteins.
2. To carry out electrophoretic separation of mammals plasma proteins.

Basic theoretical information

Electrophoresis is a method of complex mixtures separating under the action of electric field. Charged particles pass through the solution or gel, move in them, while uncharged particles are immovable in electric field.

Serum protein electrophoresis is widely used in medicine. Until then electrophoresis gels become generally accepted, protein electrophoresis was performed on paper (sometimes in combination with paper chromatography). By electrophoresis on paper and cellulose acetate films in clinical diagnostic laboratories, 5 fractions of proteins – albumins, α_1-, α_2-, β-, and γ-globulins are detected in blood serum. Albumins have relatively low molecular weight, so they are more mobile in gels or on paper. In constant electric field, they quickly move to the anode, globulins toward the anode pass smaller distance. In electrophoregram protein fractions appear as spots or bands. Before albumin small band that conforms to pre-albumin may appear. At some diseases abnormal protein bands appear that are used in diagnostics. The ratio of albumin and globulin fractions has an important diagnostic value (normal is 1.5–2.3).

The rate of movement of particles depends on the following factors:
– the charge of particles;
– applied electric field;
– temperature;
– nature of the suspended medium.

Gel electrophoresis is a method for macromolecules (nucleic acids or proteins) separation depending on their molecular weight, configuration, electric charge, and other physical properties. A gel is solid colloid. The term “electrophoresis” describes the migration of charged particles under the influence of an electric field. "Electro" refers to the electric energy, and "phoresis", from the Greek verb “phoros”, means "to carry across". Thus, gel electrophoresis refers to the technique in which molecules are forced across pores of gel, motivated by electric field. Activated electrodes at either end of the gel provide the driving force. Molecule properties (weight and charge firstly) determine how rapidly it can move an electric field through a gel.

Many important biological molecules such as amino acids, peptides, proteins, nucleotides, and nucleic acids, possess ionized groups and, therefore, at any given pH, exist in solution as electrically charged particles, either as cations (+), anions (–) or zwitter-ions (have as “+” as “–“). Depending on the nature of the net charge (positive or negative), the charged particles will migrate to either the cathode or the anode.

There are 2 basic types of materials used to make gels: agarose and polyacrylamide. The polyacrylamide gel electrophoresis (PAGE) technique was introduced by C. Raymond and L. Weintraub (1959). Polyacrylamide is similar to the material used for skin electrodes and soft contact lenses creation. Polyacrylamide gel may be prepared so as to provide a wide variety of electrophoretic conditions. The pore size of the gel may be varied to produce different molecular sieving effects for separating molecules of different sizes. By controlling the percentage of polyacrylamide (from 3 % to 30 %), precise pore sizes can be obtained, usually from 5 to 2 000 kDa (kilo Dalton). This is the ideal range for genes, polypeptides, proteins including enzymes, sequencing. Polyacrylamide gels can be prepared in a single percentage or with varying gradients. Gradient gels provide a continuous decrease in pore size from the top to the bottom of the gel, resulting in the appearance of more bands as a result of mixture (of genes, proteins, etc.) separation, i.e., mixture is separated into components better. Because of this

banding effect, for instance, detailed molecular genetic analysis can be performed with gradient polyacrylamide gel (Fig. 5).

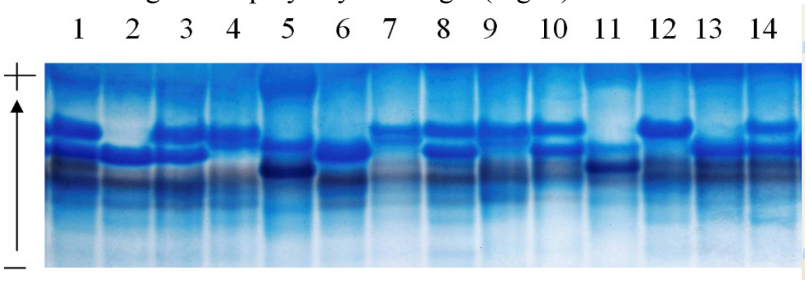


Fig. 5. Electrophoresis of carp transferrin in polyacrylamide gel: lanes 1, 3, 8, 10, 14 are of genotype AC_1; 4, 7 and 9 – AB; 11 – C_1D; 12 – AA; 2 – C_1C_1; 6, 13– C_1C_2; 5 – BD

Polyacrylamide gels offer greater flexibility and more sharply defined banding than agarose gels. SDS-electrophoresis is carried out for mixtures treated with sodium dodecyl sulfate (SDS). As all components (for example polypeptides) are "wrapped" with SDS and thus become strongly negatively charged, they migrate through the gel depending on their size (small polypeptides migrate faster than large ones). SDS-polyacrylamide gel electrophoresis is the most used in scientific research.

The discrete electrophoresis in polyacrylamide gel involves the use of gels of two types (Fig. 6):

1. Concentrating (stacking) gel (top);
2. Separating (running) gel (bottom).

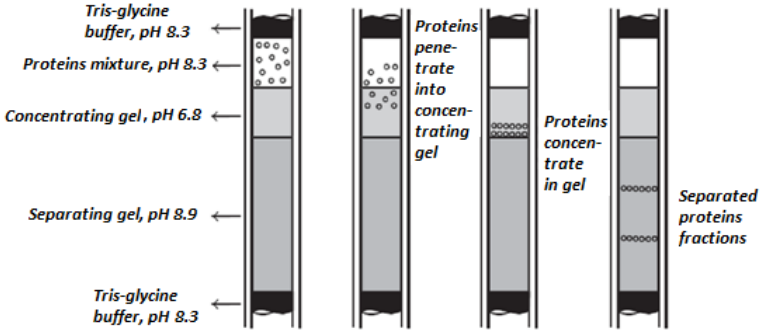


Fig. 6. The movement of proteins through gel electrophoretic system

The concentrating (stacking) gel usually contains 5 % of acrylamide and tris-HCl buffer of pH 6.8. The separating (running) gel has a high percentage (usually 10 % – 15 %) of the acrylamide.

Methods of electrophoretic separation of proteins are used to analyze composition and purity of protein fraction or complex, size (molecular weight) of protein, and for genotyping. A variety of electrophoresis - isoelectric focusing – is used to determine the isoelectric points of proteins. Immunoelectrophoresis allows exploring the antigenic composition of biological material.

Equipment, tools and materials

Camera for the vertical gel electrophoresis, power supply, micropipette, tips or micropipettes, microtubes with mammals blood plasma, beakers of 20 ml, 50 ml, 100 ml; glass rods, teflon comb, scalpel, bidistilled water.

Buffers for making gels:

Lower buffer (for the separating gel) (A) (36.4 g of tris; 0.4 g of SDS. Dilute up to 100 ml with H_2O_{dist},, adjust pH up to 8.3 using 6 M HCl).

Lower buffer (for the separating gel) (B) (28 g of acrylamide, 0.8 g of bis-acrylamide. Dilute up to 100 ml with H_2O_{dist}). Wrap the bottle with aluminum foil, as the solution is light sensitive.

Concentrating (upper) buffer (for the concentrating gel) (B). Dissolve 6.06 g of tris; 0.4 g of SDS in ~ 70 ml of water, adjust pH up to 6.8 with 6 M HCl, and then dilute up to 100 ml with $H_2O_{dist.}$

The concentrating (upper) buffer (for the concentrating gel) (B) (10 g of acrylamide, 2.5 g of bis-acrylamide). Dilute up to 100 ml with water. Wrap the bottle with aluminum foil, as the solution is light sensitive.

The electrophoretic running buffer pH 8.3 (for the tank):

Tris 121.4 g;
Glycine 567 g;
SDS 40 g;
Dilute with water up to 4 liters.

Dyes:

Amido Black – for staining gel plate for transferrin detection:

Methanol 40 ml;
10 ml of glacial acetic acid;
Amido Black 10B 0.1 g;
Ammonium persulfate (PSA) 10 g. Dilute up to 100 ml with $H_2O_{dist.}$

Coomassie blue – for staining protein bands in gels:

Coomassie Blue R250 – 1.25 g;

Ethanol 96 % – 125 ml;

Glacial acetic acid – 125 ml;

Water – 250 ml.

Bromphenol blue – to visualize the analyzed samples (marker dye).

Solution for gel laundering:

Ethanol 96 % – 100 ml;

Glacial acetic acid – 100 ml;

Water – 800 ml.

Work procedure

1. According to the instruction prepare the lower separating (running) gel:

1. Lower buffer (A) 2,345 ml;
2. Lower buffer (B) 9.166 ml;
3. Water 10 211ml;
4. Ammonium persulfate (PSA) 0.275 ml (275 ml);
5. TEMED 0,08 ml (80.0 µl) (add TEMED in the fume hood);

Total 22,077 ml.

Acrylamide in its unpolymerised form is a potent neurotoxin, so you should wear gloves when making gels, setting up the tank for running the gel, and during transferring the gel. After adding the last component – ammonium persulfate (PSA) gel starts to polymerize quickly, so it is necessary to pour gel quickly and carefully into the space between tank glasses for electrophoresis (wait for gel polymerization).

2. According to the instruction prepare upper concentrating (stacking) gel:

1. Concentrating (upper) buffer C0.5 ml (500 ml);
2. Concentrating (upper) buffer D 2 ml (2000 ml);
3. Water 2.5 ml (2500 ml);
4. Ammonium persulfate 0,04ml (40µl);
5. TEMED 0.04 ml (40 ml) (add TEMED in the fume hood);

Total 5.08 ml.

Pour carefully the solution into the space between the tank glasses, and rapidly, before polymerization starts, insert the Teflon comb between the tank glasses.

3. After polymerization, remove carefully the teflon comb from the concentrating gel in order to create wells. Fill the wells with tris-glycine running buffer.

4. Prepare aliquots of proteins. They should be of room temperature. Using a micropipette (sampler) add marker dye bromphenol blue (2µl) in each well on the tablet and add proteins (5 µl) in each well with a separate tip, mix thoroughly.

5. Using a microdispenser (micropipette) take 7 µl of the sample and transfer each sample separately in the wells of concentrating gel.

6. Pour a sufficient quantity of running buffer into both the lower and upper chambers of the electrophoresis apparatus until the bottom of the gel is immersed in buffer. Be careful not to disturb the samples in the wells when adding buffer to the upper chamber.

7. Assemble the top of the electrophoresis apparatus and connect the system to an appropriate power source. Turn on the power supply and run the gel at 20 mA constant current.

8. When the tracking dye reaches the separating gel layer, increase the current to 30 mA. Continue applying the current until the tracking dye reaches the bottom of the separating gel layer (approximately 4–5 hours).Turn off and disconnect the power supply. Disassemble the gel apparatus and remove the glasses which contain the gel. Place the glasses on paper towels and carefully remove the clamps from the sandwich.

9. Working on one side of the sandwich, carefully slide 1 of the spacers out from between the 2 glass plates. Using the spacer or a plastic wedge as a lever, gently separate the glass plates apart without damaging the gel contained within. Lift the bottom glass plate with the gel and transfer the gel to container filled with Coomassie Blue dye. After 12 hours, wash the gel of excess dye with the solution for gel laundering and observe the results of electrophoretic separation of proteins.

Processing of experimental data

Transfer the stained block on glass and perform fractions typing of transport proteins (transferrins) for each individual. Note in the workbook the genotypes of each examined individual.

Analysis of results

Basing on the results draw the conclusion on the level of heterozygosis and poliallelism, if presented in the individuals examined.

Test questions

1. Explain what the principle of vertical polyacrylamide gel electrophoresis of proteins is.
2. Which factors does the rate of movement of proteins in polyacrylamide gel depend on?
3. What is an isoelectric point of the protein?
4. What are the reasons for using polyacrylamide gel electrophoresis of proteins?

Sources: [7, 8].

Laboratory work 7

AGAROSE GEL ELECTROPHORESIS OF DNA FRAGMENTS

Aim of work – to master the technique of electrophoretic separation of DNA fragments.

Main tasks of work

1. Perform electrophoretic separation of DNA fragments of plant and animal origin.
2. Perform comparative analysis of the rate of migration of DNA fragments of plant and animal origin.

Basic theoretical information

Horizontal agarose gel electrophoresis is a method used in molecular biotechnology to separate DNA and RNA fragments of different length. Nucleic acids molecules are separated by using an electric field, causing negatively charged particles move in agarose gel toward anode. The larger molecules move more slowly through the gel while the smaller molecules move faster. Gel electrophoresis is performed in special chambers (Fig. 7).

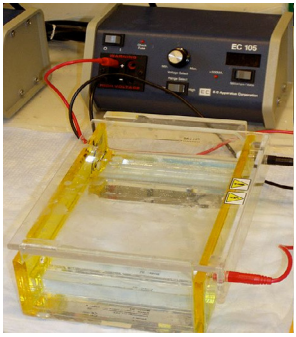


Fig. 7. Chamber for horizontal gel electrophoresis

Gels suppress thermal convection induced by an electric field and perform separation medium function (sieve). Gel electrophoresis of DNA is usually performed for analytical

purposes, often after increasing the number of specific DNA fragments by polymerase chain reaction (PCR), as well as a preparatory stage for establishing the quality of DNA isolation.

Agarose consists of long sequences of linear polysaccharides formed by alternating residuals of β-D-galactopyranose and 3,6-anhydride-α-1-galactopyranose linked by 1–4 glycoside bonds. Various samples put into adjacent wells will move parallel but at different rate depending on the molecular weight of DNA fragments. Depending on the number of different molecules in the sample, each line will show a separate component of the original mixture. Lines in the gel that are at the same distance from the beginning of the gel block contain molecules that move with the same speed, which usually indicates that they are almost of the same size. To set the lengths of fragments, markers of known size are used. There are a great number of DNA length markers, in particular such as lambda HindIII, lambda PstI, PhiX174. Markers are selected depending on the length of DNA fragments which are expected to be received. For small PCR products the PhiX174 is chosen, and lambda HindIIIis chosen for fragments of 6 thousand base pairs. At present the manufacturing companies offer markers with groups of "bands" in certain intervals: 0.5; 1.0; 1.5; 2; 2.5 thousand base pairs and so on up to 10 thousand base pairs. Agarose gel electrophoresis can be used for distribution of DNA fragments ranging from 50 base pairs up to several million. DNA fragments of different lengths can be divided using gels with different concentrations of agarose. Higher concentrations of agarose enable to conduct the distribution of smaller DNA fragments, while low concentrations of agarose allow distributing in gel large fragments of DNA. Fig. 8 shows the movement of a number of DNA fragments in agarose gel of three different concentrations, which are distributed in the same chamber by using the same voltage and the same time period.

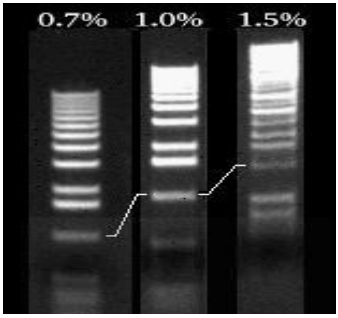


Fig. 8. The distribution of DNA fragments

Notice that large fragments are distributed much better in 0.7 % gel, while small fragments are better separated in1.5 % gel. Fig. 8 shows in each track of gel the fragment with length of 1000 base pairs.

The voltage applied to the gel increases during electrophoresis. The best

separation of DNA fragments with length of 2 thousand base pairs is achieved by the application of voltage not exceeding 5 V per 1 cm^2 of the gel.

Today several different buffers for electrophoresis of DNA fragments are recommended. For double DNA, TAE (Tris-acetate-EDTA) and TBE (Tris-borate-EDTA) are typically used. DNA fragments migrate in the gel under different conditions in these two buffer systems, as the solutions have different ionic strength. Buffers not only determine pH indicator, but also provide ions to support electro conductivity.

During electrophoresis, staining of macromolecules is performed in order to make them visible. DNA can be visualized by using ethidium bromide, which is designated as EtBr. DNA fragments moving through the EtBr gel accumulate a given dye making it possible to visualize these molecules under ultraviolet light. Fluorescence intensity depends on the amount of DNA; that is why for visualization at least 20 nanograms of DNA are necessary. EtBr is a known mutagen, so less hazardous dyes such as SYBR Green I, which is 25 times more sensitive and safer than EtBr, is used when possible.

Application of electrophoretic separation of DNA fragments:

– Evaluation of DNA molecules size after treatment with enzymes – restrictases, for example, during mapping a cloned DNA.

– Analysis of PCR products, e.g., molecular and genetic diagnosis or genetic fingerprint.

– Distribution of genomic DNA fragments for *Southern* blotting.

Equipment, tools and materials

Chamber for horizontal gel electrophoresis and power supply, gel casting tray, sample combs to form wells, transilluminator (UV light box) used to visualize ethidium bromide-stained DNA in gels, nitrile rubber gloves (latex gloves), 10 × buffer of tris-borate-EDTA (TBE) (0.9 M tris, 0.9 M boric acid, 20 mM EDTA. pH up to 8.1 – 8.2 using dry boric acid, agarose), ethidium bromide (10 mg/ml in sterile distilled water), bromphenol blue.

Work procedure

1. Prepare a working solution of buffer Tris-borate-EDTA10xTBE: 218 g of Tris; 110 g of boric acid; 9.3 g of EDTA.

2. Dissolve the ingredients in 1.9 liters of distilled water. pH indicator to about 8.3 using dry boric acid or NaOH solution. The volume of the solution diluted to 2 liters using distilled water. With

working solution prepare 1 × TBE in a volume sufficient to fill the chamber for electrophoresis and preparation of gel.

3. Add to 1 × TB agarose in quantities necessary for obtaining 0.45 % – 0.75 % solution and heat on a water bath until agarose is completely dissolved.

4. Cool down the mixture to about 50 °C. While cooling seal the edges of gel casting tray with adhesive tape (Fig. 9). Add ethidium bromide to agarose solution to a final concentration of 0.5 μg / ml and mix carefully avoiding of air bubbles in the gel.

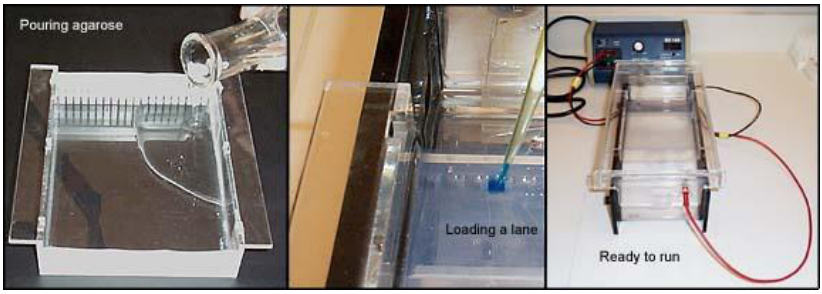


Fig. 9. Preparing gel and bringing samples in wells

5. Pour warm agarose in the gel casting tray (Fig. 9) and evenly distribute it in the gel casting tray. Insert the comb vertically so that its cloves do not touch the bottom the gel casting tray about 1.5 mm.

6. Leave the gel casting tray with agarose gel for 30 minutes, and then carefully remove the comb and the adhesive tape. Place the gel casting tray in the chamber for electrophoresis containing necessary amount of 1 × TBE with ethidium bromide at a final concentration of 0.5 μg / ml.

7. Prepare for electrophoresis the samples of studied DNA, which is mix with the buffer for applying (5:1). To get a clear signal after staining by ethidium bromide, it is sufficient to put200 ng of DNA marker to the well with the width of 5 mm. To construct a standard curve it is necessary to use marker fragment, the length of which is approximately equal to the length of the studied DNA.

8. Put carefully studied and marker DNA in the wells (Fig. 10). To improve the accuracy of DNA fragment size definition, put marker DNA into wells on both sides of studied DNA.

9. Connect electrodes of the horizontal electrophoresis chamber to a power supply and perform electrophoretic separation of DNA fragments using electric field gradient of 1–3 V per 1 cm^2 of the gel for 1–3 hours.

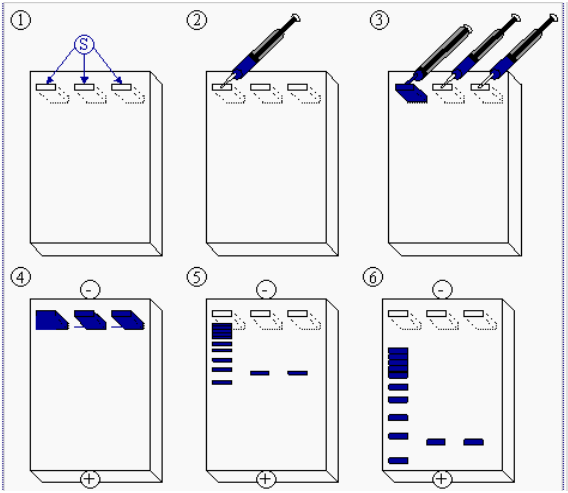


Fig. 10. Adding DNA samples in wells

Processing of experimental data

When it is necessary to check if you have succeeded in extracting the DNA from biological material, 20 nanograms of DNA sample are added to the gel with ethidium bromide. To visualize products of polymerase chain reaction 10–20 micro liters are typically used, depending on the reaction conditions.

Analysis of results

To analyze the results it is necessary to remove the agarose gel with a spatula, transfer it on the transilluminator and under ultraviolet light analyze the results of DNA fragments separation. Record the results using photo of the system and paste the image in the workbook.

Test questions

1. What are physical and chemical properties of DNA?
2. What is physical principle of electrophoretic method of biomolecules study?
3. What physical and chemical factors does effectiveness of electrophoretic separation of nucleic acids depend on?
4. What dyes and why are used to visualize the results of electrophoresis of nucleic acids?

Sources: [5, 9].

Laboratory work 8

NANOPARTICLES OBTAINING AND DETECTING

The aim of work – to master the technique of luminescent carbon nanoparticles obtaining

Main tasks of work

1. Obtain nanoparticles of carbon – carbon dots.
2. Detect the presence of carbon dots by their luminescence.

Basic theoretical information

Nanostructures have dimensions from 1 to 100 nm (1 nm = 10^{-9} m), which makes their unique physical and chemical properties. Nanotechnology is a set of scientific knowledge, methods and tools of directed regulated synthesis of individual atoms and molecules of various substances, materials and products with linear dimensions of structural elements up to 100 nm. Nano-objects include separate entities having size of 1–100 nm in one or more dimensions (nanoparticles, nanofibers, nanospheres, nanocapsules, liposomes, dendrimers, nanotubes, nanofilms etc.) and nanocomposites – materials consisting of macroscopic polymer matrix and dispersed therein nano-sized structures.

Many biological molecules are nanoscale, for example, insulin linear dimensions are about 2.2 nm, hemoglobin and fibronectin – from 4.5 to 7.0 nm, lipoprotein – about 20 nm, fibrinogen – from 5 to 70 nm (Fig. 11).

a *b* *c* *d*

Fig. 11. Biological molecules having nanoscale: *a* – hemoglobin (68 kDa, 4.5 x 7 nm), *b* – lipoprotein (1300 kDa, 20 nm), *c*– fibronectin (1 subunit – 200–250 kDa, 2–3 × 60–70 nm), *d* – bovine fibrinogen (300 kDa, 5 x 70 nm)

Quantum dots are crystalline clusters often of semiconductor materials with size of 1–10 nm in three dimensions. Carbon is a conductor in some forms. Diamond, an allotropic form of carbon, is a semiconductor.

Quantum dots are characterized by broad absorption spectra and narrow symmetric spectra of emission, relative photostability. Their luminescence lasts a few hundred nanoseconds (about 10^{-7}s). Emitted wavelength depends on the size of a quantum dot which can range from 365 to 1350 nm. Quantum dots are fluorescent probes used in biotechnology for immunofluorescent analysis, visualization of live cell, research of organisms *in vivo*.

Luminescence is radiation of photons from electron-induced states. When the substance absorbs a certain energy it becomes excited, thus its electrons move to higher energy levels. The transition of electrons from higher energy levels to lower levels is accompanied by radiation. Substances may emit if excited during chemical reactions (chemiluminescence), biochemical reactions (bioluminescence), red-ox reactions on the electrode (electrochemiluminescence).

Luminescence can be divided into fluorescence and phosphorescence, depending on the nature of radiative jump.

Phosphorescence is radiation which can be continuous in time (during 10^{-3}–10^{-2}sec) because there is a spin ban for such transition from an excited triplet state T_1toa basic singlet state S_0 ($T_1 \rightarrow S_0$).

Fluorescence is a radiation or authorized by spin emissive transition taking place when an electron is returned to a lower energy orbital, in the ground state S_0, from the lowest singlet vibrational level S_1 (transition between singlet states $S_1 \rightarrow S_0$). Substance – fluorophore – absorbs and emits light, but emitted light has bigger wavelength, that is lower energy. The shift of emission spectrum relative to excitation spectrum is called the Stokes shift (Fig. 12).

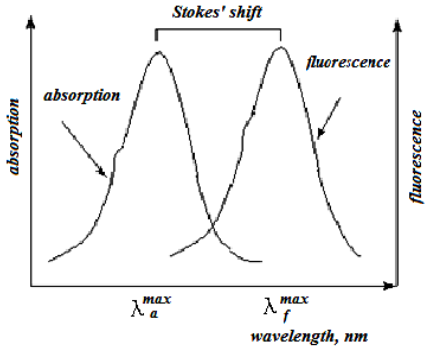


Fig. 12. Typical absorption and fluorescence spectra

High values of emission rate lead to a very short time of fluorescence decay – 10^{-11}–10^{-6}s. The lifetime of fluorescence is an average period of time during which a fluorophore is excited.

The processes of light absorption and emission are illustrated in Jablonski diagram (Fig. 13). Transitions between states are shown by vertical lines.

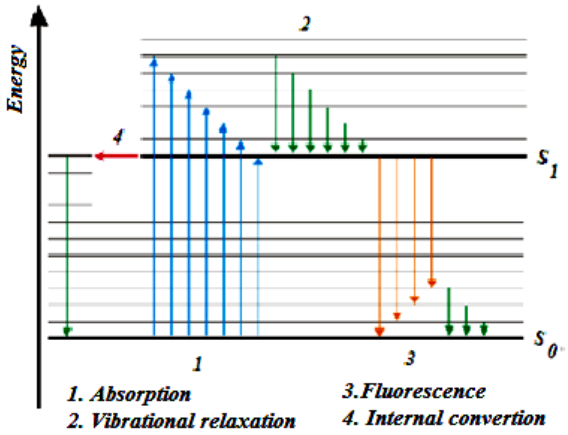
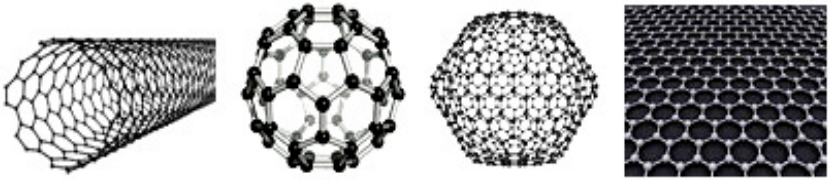


Fig. 13. Jablonski diagram

Electrons of quantum dots have some fixed energy levels with typical distances between them. During transition between energy levels quantum dots can emit photons. Changing the size of quantum dots, it is possible to change the frequency of transitions, that is, to obtain luminescence with different wavelength.

Carbon fluorescent nanoparticles are different structures including short fragments of graphene, graphene oxide, nanotubes, carbon points (Fig. 14).



a *b* *c* *d*

Fig. 14. Carbon nano-objects: *a* – single-wall nanotube; *b* – hollow polyhedron of regular form – fullerene C_{60}; *c*– fullerene S_{540}; *d* – allotropic modification of carbon –graphene

Carbon dots (C-dots) are different as to their method of creation, composition and chemical modification of their surface. They are quite cheaply synthesized, have a stable fluorescence in water medium, and are not toxic. Therefore, they can be an alternative of traditional luminophors.

Carbon dots are obtained by hydrothermal treatment of various organic compounds, such as citric acid, alanine, glycerol, sucrose, and many others. Depending on the method of obtaining and solvents used, carbon dots differ by their fluorescent properties. Thus, when using distilled water, the peak of fluorescence shifts towards ultraviolet spectrum range if compared with the solvents, which have a significant amount of calcium, potassium and magnesium salts.

For carbon dots luminescence spectrum dependence on the size of nanoparticles is not evident, but fluorescent carbon nanoparticles obtained by different methods have different emission spectrum.

Equipment, tools and materials

Citric acid, thiourea, alanine, crystalline sucrose; distilled water, ethanol, glycerol, Ringer's solution (pH 7.4), polyethylene glycol (PEG-150), glass rods, spatulas, porcelain crucible of 20 ml, glass of 100 ml, laboratory scales, black light lamp (Delux EBT-01 26 W), electric oven, microwave oven, laboratory centrifuge, centrifuge and ordinary tubes, dark room.

Work procedure

For obtaining carbon dots with blue fluorescence, perform the following:

1. Citric acid quantity of 0.21 g (1 mmol) and thiourea of 0.23 g (3 mmol) dissolve in 5 ml of distilled water.

2. Transfer the transparent solution into a porcelain crucible, heat in an electric oven at a temperature of 180 °C for two hours to form a black substance.

3. Transfer quantitatively the obtained product to a centrifuge tube, washing off 10 ml of ethanol.

4. Centrifuge the liquid at 5 000 rpm for 5 minutes. Reject the black sediment. Pour the supernatant into another tube. Ethanol contains carbon nanoparticles.

5. Place the tubes in the drying oven at a temperature of 75 °C for several minutes to evaporate alcohol. Carbon nanoparticles deposit on the walls of the tubes.

6. Wash off nanoparticles from the walls of the tube with 3–5 ml of distilled water, fill the pipette with the liquid several times, and then pour it with force on the walls, thus thoroughly mixing the content.

7. For the detection of carbon dots by their fluorescence the content of the tube irradiate with a black light lamp (wavelength is 360 ± 15 nm). During irradiation, carried out in the dark, observe blue fluorescence of carbon dots.

For obtaining "purple" carbon dots dissolve 0.5 g of alanine in 2 ml of distilled water and treat in the microwave (600 W) for 1.5 min. The resulting substance dilute with distilled water and then subject to several centrifugations at 5 000 rpm for 5 minutes. Collect the supernatant, irradiate with a black light lamp in the dark, and observe violet fluorescence.

For obtaining "blue" carbon dots mix 5 ml of glycerin with 3 ml of Ringer's solution (pH 7.4) and 2 ml of distilled water, treat in a microwave oven (700 W) for 6 minutes. The resulting product dilute with distilled water and then subject to several centrifugations at 5000 rpm for 5 minutes. Collect the supernatant, irradiate with a black light lamp in the dark, and observe blue fluorescence.

For obtaining "green" carbon dots mix 4 g of sucrose with 0.5 g of PEG-150 and add 26 ml of distilled water. Collect the sample and treat it in a microwave oven (600 W) for 4 min. Then add the sample is to another 30 ml of distilled water for its dissolution, centrifuged for 5 000 rev/min for 5 minutes. Collect the supernatant, irradiate with a black light lamp in the dark, and observe green fluorescence.

Processing of experimental data

Draw schematically probable excitation and fluorescence spectra of obtained carbon nanoparticles (carbon dots, C-dots).

Draw a diagram showing compliance of light wavelength with certain color. Mark what nanoparticles by fluorescence color you have received.

Analysis of results

Explain what determines the spectrum of quantum dots luminescence. Does the spectrum of luminescence of carbon nanoparticles depend on their size? How to receive carbon dots with blue, purple, dark blue fluorescence?

Excitation, emission and absorption spectra can be recorded automatically with spectrophotometers and spectrofluorimeters with

devices that build diagrams. For example, using a spectrophotometer LambdaBio (PerkinElmer) and spectrofluorimeter QuantaMaster (PTI) such spectra for obtained carbon nanoparticles were recorded (Fig. 15).

Give feasibility of using carbon dots as fluorescent probes.

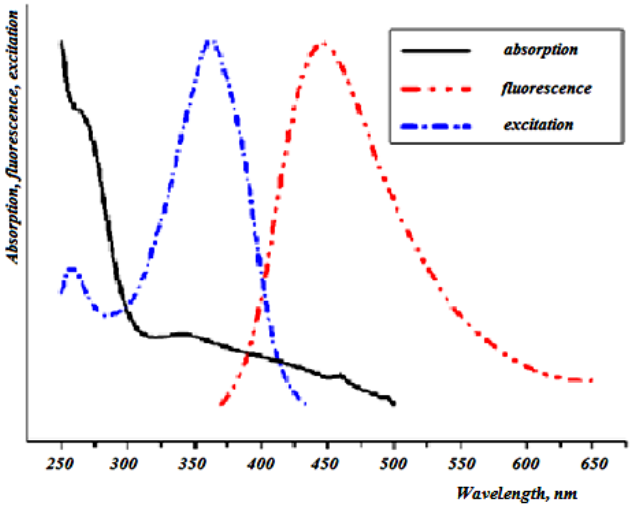


Fig. 15. Excitation, emission and absorption spectra of carbon dots

Test questions

1. What structures belong to nanostructures?
2. What is nanotechnology?
3. What biological molecules are of nanoscale?
4. What carbon nanoparticles do you know?
5. What nanoparticles are called quantum dots?
6. How to obtain a carbon dot?
7. What is luminescence?
8. What is the difference between phosphorescence and fluorescence?
9. What is the Stokes shift? What Stokes shift is characteristic to fluorescent probes?
10. What does Jablonski diagram show?
11. How can carbon dots be used?

Sources: [10–14].

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Appendix 1

Establishment
Legal address
Establishment unitwhere research was performed

CONCLUSION OF MOLECULAR AND CYTOGENETIC DIAGNOSTICS

PATIENT NAME AND SURNAME: _____

Date of birth_____

Sent by:_____

Clinical diagnosis* *Prader-Willi Syndrome*_____**

Cytogenetic diagnosis _____* *46,XX*_____**

Biological specimen: * *peripheral blood lymphocytes*_____

Research was performed with molecular and cytogenetic FISH method using *locus-specific DNA-probes LSI PWS/AS Region Probe – LSI SNRPN Spectrum Orange /CEP15 D15ZI Spectrum Green /PML Spectrum Orange (Vysis).*

Clinical conclusion:* *46,XX.ish 15q11~13 (SNRPN×2)*

Interpretation of findings: * *absence of SNRPN deletion*

Recommended*molecular diagnosis of disomic chromosome 15.*

Analysis performed by:

_____
_____

Date of analysis:_____ 20___.

* sample information

Навчальне видання

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для студентів спеціальностей 7/8.05140103
«Фармацевтична біотехнологія»

(Англійською мовою)

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