VERONIKA **BORBÉLYOVÁ et al.**

BASIC LABORATORY METHODS OF BIOMEDICAL RESEARCH

COMENIUS UNIVERSITY BRATISLAVA

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Basic Laboratory Methods of Biomedical Research

Textbook for Practical Courses of the IMBM Summer School of Biomedicine 2020-2022

for High School Students and University Students

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Comenius University Bratislava

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PREFACE

This **textbook** is intended for high school and university students of natural and medical sciences, and was created as a supplementary textbook to the practical courses of the Institute of Molecular BioMedicine (IMBM) Summer School of Biomedicine from 2020 to 2022, which was implemented thanks to the grant support of the Cultural and Educational Grant Agency of the Ministry of Education, Science, Research and Sport of the Slovak Republic: project no. **045UK-4/2020**, project title: **Summer School of Biomedicine for High School Students and University Students**.

The goal of the IMBM Summer School of Biomedicine was to familiarize high school and university students with biomedical research and to teach them the basic laboratory methods in the laboratories of the IMBM. Students have an opportunity to try out research work in our laboratories within the premises of the Faculty of Medicine, Comenius University, and in the Pavilion of Medical Sciences of the Slovak Academy of Sciences by participating in ongoing IMBM projects. This **textbook** aims to summarize basic practical knowledge of selected molecular, biological, biochemical, and imaging methods with brief protocols, which were implemented during the IMBM Summer School of Biomedicine.

The introductory chapters are devoted to the elementary skills that are necessary for successful laboratory work, such as basic lab rules, weighing of samples on analytical scale or correct and precise pipetting which are fundamental steps in the life of a researcher in any scientific laboratory. In the next chapters, the authors describe tips and tricks for searching for relevant publications in the most commonly used databases and also make suggestions on how to properly design an experiment. In general, researchers work also with laboratory animals, and for that reason, we focus some chapters also on basic manipulation with laboratory rodents, from evaluating the behavior to measuring metabolic parameters in laboratory rats and mice. As students of the IMBM Summer School of Biomedicine had an opportunity to do within the projects of IMBM molecular (e.g., polymerase chain reaction (PCR) method, isolation of DNA and RNA) and also in vivo imaging methods (e.g., micro-computed tomography) the next chapters deal with an explanation of these methods. The last part of this textbook is devoted to the proper collection of data into Excel and also basic statistical analyses, both of which have a key role in the life of a scientist.

1 Work in laboratories of IMBM (Veronika Borélyová)

1.1 Laboratories of IMBM

IMBM is a research institute of the Faculty of Medicine of Comenius University in Bratislava, which, together with the Biomedical Center of the Slovak Academy of Sciences (SAS), has a joint workplace in the pavilion of medical sciences SAS (Fig. 1).

Thus, summer school students have the opportunity to learn within IMBM laboratories at both Medical faculty and SAS. The IMBM laboratories are equipped to carry out biochemical and molecular-biological analyses of samples. In addition, we have several rooms for the implementation of animal experiments. Thanks to the joint workplace at SAS, summer school students have an opportunity to learn other sophisticated methods such as cytometry, confocal and fluorescence microscopy, as well as in vivo imaging methods. The variety of methods as well as the equipment of the IMBM is a unique opportunity for summer school students to learn basic methods of biomedical research.

The standard working hours of IMBM are from 8 a.m. until 4. p.m. However, we are so flexible regarding working time as some of the projects require more time per day or even work in the laboratory during the weekends as well. Summer school students can work in our laboratories just under the supervision of Ph.D. students or postdocs all the time.

Working in a science lab in general can be a potentially dangerous place, as scientific projects require working with chemicals, or other numerous hazards: biological, physical, and even radioactive or other risky procedures. The importance of lab safety cannot be emphasized enough. Having a set of overall laboratory safety rules is essential to avoid accidents in the lab. If students are not careful and do not follow the basic lab rules, they could sustain an injury or also damage our lab equipment. Thus, we have a list of lab rules (chapter 1.2), which students have to follow during their stay at our laboratories to ensure their safety and the safety of others.

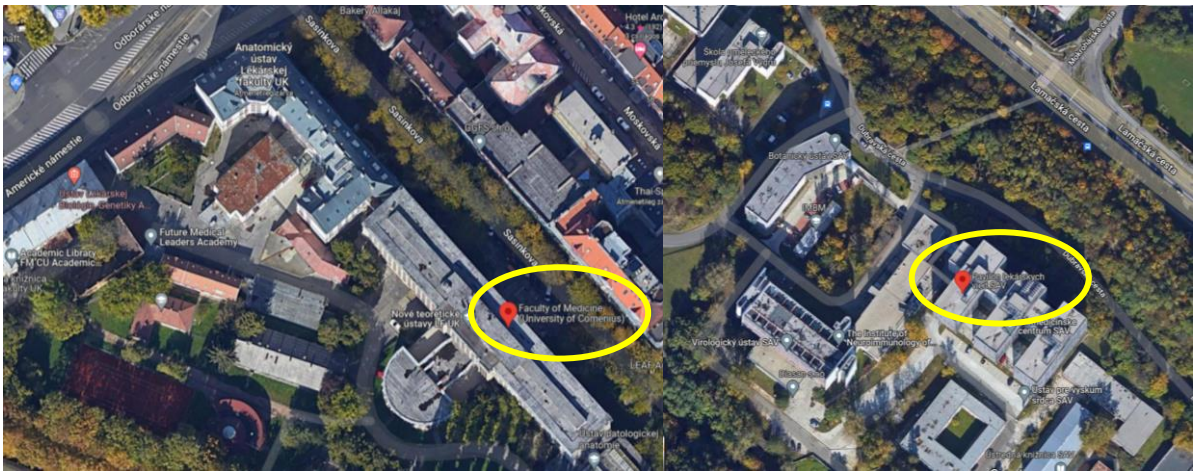


Fig. 1 Laboratories of IMBM.

Faculty of Medicine	Pavilion of Medical Sciences
Comenius University in Bratislava	Slovak Academy of Sciences
Sasinkova 4	Dúbravská cesta 9
811 08 Bratislava	845 05 Bratislava
5th floor	block B, 6th floor

1.2 Basic lab rules at IMBM

1. **Follow the instructions!**

Please, always follow the instructions of your supervisors (postdocs, Ph.D. students) while you are in the lab. Thus, it is important to listen and pay attention all the time. In addition, if you are doing some sample analysis, follow the instructions of the manufacturer and be familiar with all the steps of sample analysis. If you are not sure about any of the steps, please, get them answered before you begin.

2. **Do not eat, drink, or smoke in the laboratory!**

Do not eat or drink in the lab, there is too much risk of contaminating your food with chemicals used in the lab, and vice versa there is also a chance that your food or drinks will contaminate the experiment. At the Medical faculty, you can eat in the secretary room or on the balcony, while at SAS we also have a kitchen. At both places, you have a separate fridge and microwave for your food and drinks. Please, do not store your food or beverages in the same fridge that contains chemicals, or cell cultures.

3. **Dress for the lab!**

If you are going to the lab, you need to be dressed properly from head to toe. Thus, the first thing is to change your **boots** to covered shoes and put on **lab coats**. We have blue uniforms or standard lab coats available for you, but if you prefer your lab coats, you can bring them to the lab. If you have **long hair**, keep it tied to avoid contaminating your experiment or flame. If you are going to work with chemicals or prepare solutions, you should also wear **gloves** or even **safety goggles**. Wearing protective attire not only reduces the risk of damage to the skin and eyes but also minimizes the possibility of contamination of your experiment.

4. **Handle the chemicals carefully!**

Injuries from chemicals in laboratories are very common, and if you do not work with chemicals accurately, they can be even fatal. You should not smell or taste chemicals or biological cultures in the lab. Pay extreme attention when mixing chemicals, hold the bottle used for mixing away from your body and swirl it gently. To avoid accidents, label the bottles or containers with the name of the chemicals you are doing with.

5. **Dispose of the lab waste appropriately!**

Some waste created during laboratory work has to be disposed of in another way than just throwing it into a regular waste bin. In general, laboratories are equipped with separate designated bins or containers for disposing of chemicals, or sharp laboratory products (needles, razors, or laboratory glass). If you are working with biological cultures, make sure, that it is safe to clean up with soap and water or that a stronger agent is needed to destroy dangerous microorganisms present in the cultures (e.g., Savo). If you are not sure, please, ask Ph.D. students or postdocs.

6. **Leave experiments at the lab!**

For your safety, but also for the safety of your colleagues you are working with, leave your experiment in the lab, thus, do not take lab equipment home with you.

7. **Always keep your work area clean in the lab!**

You have to disinfect the working area(s) before and also after using them. If you are working in the lab, you have to have at the desk only the materials and equipment that you need for your current work. Any other material should be stored safely in its place. After you finish your work in the lab, you have to make an order and disinfect the used area. Never leave your mess for the next person to clean up!

8. **Do not panic in case of lab accidents and know what to do if it happens!**

Even if you pay attention and work carefully in the lab, accidents do happen. If it happens, the first thing is that you do not start to panic, it can only worsen the situation. The basic rule is that at the moment of an accident, you are going to call your supervisor or Ph.D. student about what happened. Do not lie about it!

In case of fire, you have to know where the safety equipment is (fire extinguisher). In case you cut your finger, expose your skin to chemicals, or were bitten by laboratory animals you have to wash the exposed area with water and disinfect the exposed area if necessary. In case of a chemical splashing into your eye, immediately flush it with running water for at least 20 minutes.

9. **Never use lab equipment that you are not approved or trained by your supervisor to operate!**

During summer school you will do with many types of laboratory machines from pipettes up to centrifuge, and flow cytometry. You can use and do with that equipment only in presence of your supervisors or Ph.D. students, never alone.

10. **Tell your supervisor about the failure of any laboratory equipment!**

If an instrument or piece of equipment fails during use, or it is not operating accurately, tell your supervisor or lab technicians. Never try to repair an equipment problem on your own!

11. **If you are the last person to leave the lab, make sure to lock all the doors and windows and turn off all laboratory equipment** (e.g., centrifuges, ice maker, etc.)**!**
12. **Do not play a mad scientist in the lab!**

Work responsibly in any of our laboratories, thus, do not mix chemicals to see what happens. Your unresponsiveness could lead to explosion, fire, and release of toxic gasses.

13. **Do not experiment on yourself!**

Many science fiction films start with a scientist who is experimenting on himself. You will not gain a superpower, but you will be in real danger.

2 Communication with your supervisor and co-workers in the lab (Veronika Borbélyová)

Communication has an important role in our life in general. Proper and effective communication with your supervisor and colleagues results in good working relationships, that in turn minimize misunderstandings and maximize work efficiency. It also makes it easier to solve issues collaboratively in the lab. However, to achieve the above-mentioned successful relationships and a good work environment you need to practice your communication skills. Proper communication with your supervisor and colleagues (Ph.D. students, other summer school students), has a key role in successful lab work during summer school. Here are some ways to communicate properly with your supervisor and colleagues at the lab within the IMBM Summer School of Biomedicine:

- **Greet everybody!**
- **Always be polite!**

When you are communicating with your supervisor, Ph.D. students, and any other students and employees at IMBM, always be polite, and use the "magic words": please and thank you.

- **Briefly explain what you need!**

If you need an explanation of an e.g. method in the lab, or you are not sure about one step of the analysis of samples and you need to talk to your supervisor or Ph.D. student in our laboratory, make sure you know what your question is, or what exactly needs to be discussed with them. We suggest writing down all the questions you want to communicate before the conversation.

- **Choose a preferred way of communication!**

Ask your supervisor or Ph.D. students what type of communication they prefer. This is important as some people can effectively communicate via email, while others prefer to have face-to-face discussions, text using social media, or talk on the phone. Use the communication method that your supervisor or Ph.D. students prefer during the IMBM Summer School of Biomedicine.

- **Plan your conversation with your supervisor or Ph.D. students!**

You have to pay attention to not disturb your supervisor or Ph.D. students at the time they are working on something, and they need to be focused on their work. Therefore, you have to be polite and ask them when they will have time for you. Do not disturb them when they are under pressure because of several deadlines or when they are resolving an urgent problem or issue. If your supervisor or Ph.D. student prefers face-to-face discussions, plan a meeting with them ahead and prepare your questions appropriately.

- **Be concise and straightforward!**

If during the IMBM Summer School of Biomedicine, you are going to have the first meeting with your supervisor or Ph.D. student (in person or via email) you have to introduce yourself, and afterward introduce your concerns or issues that need to be solved. Keep the communication as short, simple, and direct as possible. Please, avoid giving them your complex explanations and thoughts regarding the discussed issue. If you need acute assistance on a project you are working on, please, do not wait until the last minute to ask for suggestions or instructions.

If you communicate with your supervisor or Ph.D. student via email it is suggested to keep your email as short as possible. Please, write one or maximally two paragraphs to prevent them from being boring or just skipping the main part of your message. If you want to discuss several issues, make bullet points or subheadings to make your email clear.

- **Practice active listening!**

If you have a meeting with your supervisor or Ph.D. student regarding your summer school project, be an active listener instead of worrying about your answer to their possible questions. Active listening shows that you are interested in what your supervisor or a Ph.D. student says and also you express your respect to them. While discussing your project or any issues, write down your notes to remember more from your conversation. While they are explaining something to you, do not interrupt them, it is impolite!

- **Meet regularly with your supervisor during IMBM summer school!**

During the IMBM Summer School of Biomedicine, you will communicate with your supervisor and Ph.D. students regularly. At IMBM, postdocs and also Ph.D. students are flexible regarding communication and discussion. Thus, you do not have to be afraid of talking about negative feelings as well. We can only improve things or some issues, if we talk about them and just afterward, we can solve them properly.

- **Inform us about changes in your attendance!**

In general, during the IMBM Summer School of Biomedicine, we are responsible for you. Thus, if you feel sick or you are ill, you have to let your supervisor know (postdoc and also Ph.D. student) via phone or email.

- **Write a report every week!**

Another way of communicating with your supervisor and Ph.D. students is by writing a weekly report. The meaning of the report is to have an overview of the work you did during a week, it should be a replacement for the lab book and a safe place for all data that might disappear from your computers. At IMBM we have a guideline for report writing, which is valid for every student who is working at IMBM, but also for postdocs not able to attend the weekly meeting or if the head of the institute is not present. The students receive feedback for their weekly report either from their supervisor or Ph.D.

students, who in their response can also ask some questions from the student. In that case, please **respond to their questions and do not leave them unanswered**.

The guideline for report writing is as follows:

1. Write and send the report every Friday to give all the needed information about the past week.
2. Write the reports including all attachments in English. Include the title of Your thesis and the name of Your supervisor in the subject line.
3. Write what you have done during that week – methods, but also why you have done it – the rationale and what you have expected – hypotheses.
4. Mention what new methods you have learned. Add them to a list of methods that you already can do independently at the end of the report.
5. Attach an excel file or GraphPad with raw data, a PowerPoint file with results in graphs, pictures, tables, and also the latest versions of manuscripts or theses.
6. Interpret your results in the text of the email.
7. Add a link to at least one Jove video that you have watched and at least one article that you have read. Choose both wisely, they should be recent, relevant to the topic of interest, and published in a good journal. Explain your choice and try to summarize the take-home message for your research – methods, results, or hypotheses, and try to comment on them critically. The summary should be usable for the attached thesis.
8. Evaluate your week on a scale of 0 (worst) – 10 (best) according to your plans and expectations. Explain the evaluation. Try to summarize what you have learned and what you have discovered during the last week.
9. Write your plans for the next week. Be detailed about the experiments that you want to conduct.
10. Be polite, comprehensive, and concise. Send the report to your supervisor, cc to imbm@imbm.sk, and anyone else who you were working with. Read your report again before sending it.

3 Gaining skills in basic lab techniques (Veronika Borbélyová)

3.1 Weighing of samples

Knowledge and skill regarding the weighing of samples are one of the first things that scientists learn in the lab. It is a skill that most scientists need during their careers. If you want to be accurate in weighing laboratory samples, the first step is that you need to have the proper kind of scales. For example, many weightings are rough and need just a simple top-loading scale. However, if you need to weigh semi-micro quantities of samples you must weigh the samples using an analytical scale. The accuracy and precision in weighing laboratory samples depend not only on the reliability of the weighing system but also on the skill of scientists in using them.

At IMBM you will work with two types of scales:

1. **Precision (simple) scale (Fig. 2 A):** this allows a rough weighing when you can weigh the material manually by carefully placing it in the center of the weighing plate. As soon as a stable weighing value is reached the weighing value can be read. However, this type of scale is not appropriate for dynamic weighing, e.g., when a small amount of material is removed or added to be weighed. On the other hand, you can move the scale from one room of the lab to the other room.
2. **Analytical scale (Fig. 2 B):** this type of scale allows us to measure even the slightest tiny fluctuation in sample weight. However, this type of scale is more sensitive to environmental factors, e.g., even undetectable air currents around a sample can alter reading and you cannot relocate it.

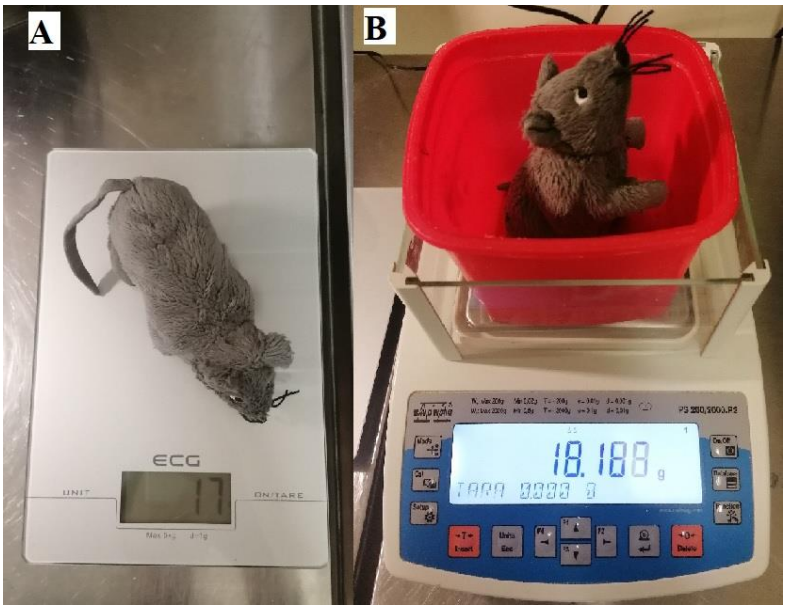


Fig. 2 Scales at IMBM. A – precision scale, B – analytical scale. Photo by Veronika Borbélyová.

Whether you use a precision scale or an analytical scale, there are several basic rules for accurate weighing:

1. Do not handle objects to be weighed with bare hands when positioning them on the weighing pan. Use gloves or paper towels.
2. Do not weigh chemicals directly on the weighing pan. Use a tare container or filter paper.
3. Do not weigh hot or cold objects on the balance.
4. Keep the weighing chamber (in case of analytical weight) or weighing pan clean.
5. Do not overload any type of scale.
6. Before using the scale, ensure that the weighing pan is clean. If it is dirty, say it to your supervisor and brush the weighing pan down with a special brush.
7. If you are working with an analytical scale, ensure that the bubble in the level indicator is centralized while the balance is "OFF".

When you are working with **an analytical scale** pay attention to **environmental factors** which could affect the analytical weight and lead to an incorrect weighing of samples. These environmental factors are as follows:

- **Temperature and humidity** – avoid exposing your balance to sunlight and infrared radiation emitted by lamps or heaters. Keep the temperature and relative humidity as constant as possible.

- **Vibrations** – you have to set up the analytical scale in a vibration-free location, as any vibration can affect the readings. Ensure that near the analytical weight are no machines that generate vibrations.
- **Air drafts** – the location of the analytical scale should be only slightly ventilated. Exposure to drafts needs to be avoided. The air flow rate should be below 0.2 m/s.
- **Magnets** – magnetism has to be ruled out, e.g., table on which the analytical scale is located may not be made of stainless steel.
- **Inappropriate handling** – you have to carefully place the tare container on the weighing pan without any force. Do not lean on the table or rest your arm on it during the weighing procedure.

3.2 Labeling of samples

Labeling systems in the scientific field is extremely important. Labeling systems we use when we label:

- bottles during the preparation of solutions,
- containers in the lab,
- samples for storage (e.g., Eppendorf and falcon tubes, deep wells, microtitration plates, storage boxes, blood collection tubes, or other types of vessels).

The durability of the labels is of prime importance. You can use:

- **temporary labels** – e.g., using a marker to pre-label EDTA or LH tubes before blood collection,
- or **permanent labels** – e.g., used for storage of plasma samples at -20 °C.

The content of a laboratory label for storage of plasma samples in **Eppendorf tubes**:

- Acronym of experiment/clinical study (e.g., Prenatal COLA)
- Date of sample collection (25.1.2023),
- Type of sample (e.g., urine, plasma, or type of organs) – here you can add whether the blood was taken into heparinized (LH) blood collection tubes or tubes with EDTA (e.g., LH_plasma),

- Group, sex of patient/animal (F=female, M=male) with the ID of patient/animal (COLA_F_ID1, CZRL_M_ID3).

The content of a laboratory label for storage of these plasma samples in **eppies in a cardboard storage box** (Fig. 3):

- Acronym of experiment/clinical study,
- Year,
- Sample type,
- Organ,
- Type of sample analysis,
- Thawed – date,
- Notes – here you can add any additional information regarding the samples,
- Name of the responsible person.

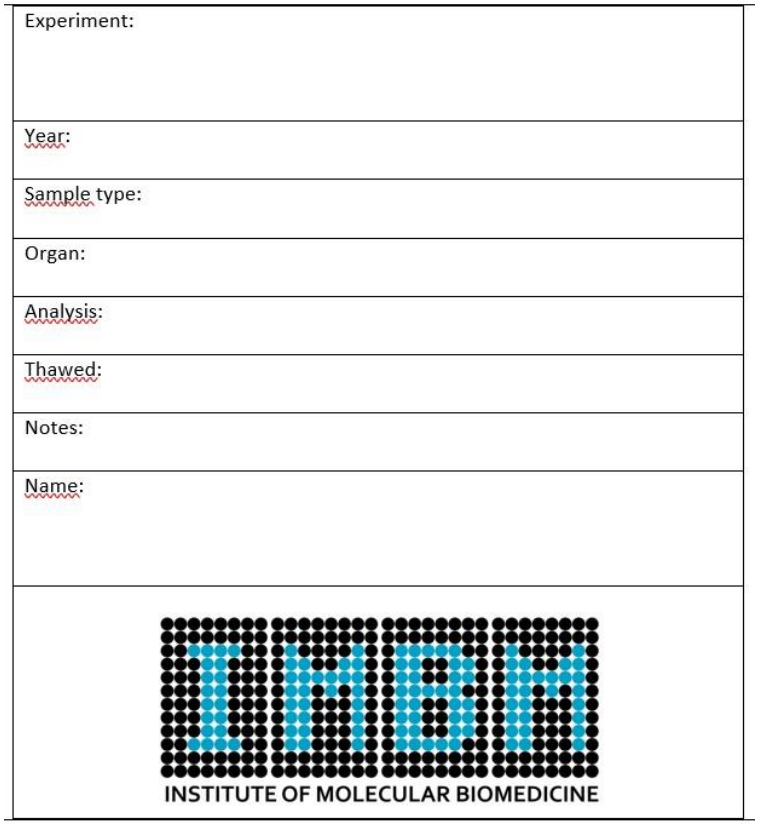


Fig. 3 The label of a cardboard box at IMBM.

Another form of sample labeling is if you pipette plasma samples to microtitration plates or deep wells. The microtitration plate typically has 6, 12, 24, 48, and 96 sample wells. Each well of those plates typically holds between tens of nanolitres to several milliliters of liquids. For example, if you

want to store urine samples from laboratory rats, a 96-well deep well plate with a maximum of 2 ml of sample is suggested. In case you know ahead that you are going to use plasma samples from laboratory rats for measuring testosterone concentrations in plasma, you can pipette plasma samples to microtitration plates with a maximum of 300 µl.

In both types of plates, the labeling is a two-step process. First, you have to know that in each well of the plates which animal or patient sample is pipetted. For this reason, you have to use a so-called pattern even in paper form, and also in excel sheets (Tab. 1). The next step is to label the plates themselves, mainly the lid of the plates that can be either a plastic lid or a transparent tape. In that case, the content of the label should be written by a permanent marker, and the information about the project, such as:

- Acronym of experiment/clinical study,
- Year,
- Sample type – e.g., LH plasma (and the way of centrifugation: 4 °C, 10 min, 1600g),
- The name of the responsible person should be reported.

Tab. 1 An example of a version of pattern for plasma samples.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A												
B												
C												
D												
E												
F												
G												
H												

3.3 Pipetting (Monika Janíková)

The most basic tools we can find in research laboratories are pipettes or micropipettes. Researchers use them daily for precise measurement and transfer of different solutions, chemicals, and samples. Vendors and suppliers are offering many different types of pipettes – automatic, manual, or electronic pipettes, and programmable pipettes which can be single or multichannel or disposable glass and plastic pipettes. Moreover, they offer multiple sizes, but generally, micropipettes are available in three sizes – 2-20 μL, 20-200 μL, and 200-1000 μL. For every student it is very important to learn how to choose and use a pipette correctly and how to pipette precisely. For purposes of Summer School at IMBM, this part will be focused mostly on manual automatic pipettes, multichannel pipettes, and an explanation of appropriate pipetting.

3.3.1 Manual automatic pipettes

The manual automatic pipette is operated by the pressing plunger while the required volume is measured by the pipette system automatically according to its previous setting. A typical single-channel automatic pipette consists of a piston button, which mechanically controls the pipette. The body of the pipette serves as a holding and mechanical volume controller for setting the pipetted volume within a certain range, which is displayed on the numerical volume indicator. The ejector of the plastic extension is used to remove the disposable plastic tip.

3.3.1.1 Forward pipetting

Before starting your work and pipetting, make sure you cleaned your working space and prepared all samples, reagents, and materials you need. For pipetting, you will need a pipette, a box of tips suitable for the size of the pipette you chose, clean tubes (for transferring your samples/reagents, etc.), a waste bin, and gloves.

1 Set the volume on the pipette to the desired volume.
Turn the volume adjustment knob clockwise or counter-clockwise until you see on the volume indicator the volume you need to set. It is very important to stay within the volume limits of your pipette based on its size. Using the wrong size of the pipette and pipetting incorrect volumes can lead to breaking the pipette.
2 Depress the plunger to the first position.
The pipette plunger has two stop positions. Before you start using the pipette, try to press the pipette from ready to the first and second positions so you will get a feel of these stops.
3 Immerse the tip to the correct depth (this depends on the pipette and tip, and it can vary) and let the plunger go smoothly to the ready position, wait a second for the liquid to flow into the tip. Try to avoid aspirating bubbles. In case you have bubbles in your tip, repeat aspirating.
4 Put the pipette against the wall of the tube where you want to transfer your desired volume under a 10-45° angle and smoothly depress the plunger to the first position – wait a second and depress the plunger to the second position.
5 Take out the pipette with the empty tip from the tube.
6 Now, let the plunger return to the ready position.
7 Discard the tip with the ejector button to the waste bin.
8 Place the pipette in a vertical position to the pipette holder.

3.3.1.2 Reverse pipetting

This technique is recommended especially for precisely pipetting small volumes, for pipetting solutions with high viscosity or a tendency to foam. Reverse pipetting eliminates the risk of foaming, sample splash, or bubble formation.

1 Clean your workspace and prepare all materials for pipetting.
2 Set the volume on the pipette to the desired volume.

3 Depress the plunger to the second position.
4 Immerse the tip and let the plunger go smoothly to the ready position and wait a second for the liquid to flow into the tip.
5 Withdraw the tip from the liquid, touching it against the edge of the tube to remove excess liquid.
6 Put the pipette against the wall of the tube where you want to transfer your desired volume under 10-45° angle and smoothly depress the plunger to the first position – hold the depressed plunger in this position.
7 Some liquid will remain in the tip, and this should not be dispensed!
8 Take out the pipette with the tip from the tube.
9 Remaining liquid in the tip can be pipetted back into the original solution or thrown away with the tip.
10 Now, let the plunger return to the ready position.
11 Discard the tip with the ejector button to the waste bin.
12 Place the pipette in a vertical position to the pipette holder.

3.3.2 Multichannel pipettes

A multichannel pipette is a specific type of automatic pipette that has more channels and therefore it is possible to pipet several wells in a plate at once, which increases productivity and reduces time. Faster pipetting reduces the interval between the pipetting solution to the first sample and the last sample. The pipette is available in 8-, 12-, and 24-, but also 96-channel versions, manual and electronic, and with different sizes of volume. With this type of pipette, it is important to visually check if the aspirated volume is the same in all tips. If the volume in some tips is different or air bubbles are present, repeat the aspiring of solution to the tips.

3.3.3 Electronic pipettes

The electronic automatic pipette is the ideal tool for accurate and reproducible pipetting when processing and analyzing a larger number of samples. The principle of their use is the same as manual automatic pipettes. Electronic pipettes are available in several formats, the most used are 1- and 8-channel pipettes (available, although less used, are 12- and 24-channel versions). The volume and the speed is set on the pipette display in pipetting mode. The degree of pipetting speed is set according to the type of pipetting fluids. If it is a viscous liquid, the pipetting speed must be set to slower. Setting up and using an electronic pipette is relatively simple and intuitive. Individual modes are selected with a rotary switch, and parameter selection and its values are set with the side buttons and saved with the main central control selected values.

3.3.4 Serological pipettes

Serological pipettes are used in tissue and cell cultures. During tissue and cell culture experiments it is necessary to ensure and maintain the sterility of used tools and solutions. These pipettes are therefore sterile and individually packed. They are easy to handle, and they are optically clear glass or plastic. They are in different sizes from 0.1 μL to 100 ml. Volume is marked on these disposable pipettes in both directions of the scale, from the top of the pipette downwards and vice versa for easier monitoring of the pipetted volume. At the end of the serological pipette is a filter. It is important to avoid getting liquid into the filter. An electronic pipette controller is a system for controlling the aspiration (button-up) and dispensing (button-down) of liquid in a serological pipette.

3.3.5 Pasteur (transfer) pipettes

Pasteur pipettes are disposable single-channel plastic pipettes in volume size 1-5 ml. Most of them are sterile and individually wrapped. The liquid is aspirated to the pipette by pressing and gradually releasing the plastic balloon, which is an integral part of the pipette. This creates a negative pressure in the pipette and the liquid is aspirated into the pipette. They are used in laboratories when the transfer

of a small amount of liquid is required, and it is not possible to use automatic pipettes. They are ideal for pipetting potentially infectious samples as well as toxic substances. The advantage of these pipettes is their low price, availability, and simplicity of use. Their disadvantage is that only an approximate volume can be measured and pipetted.

3.3.6 How to avoid contamination in pipetting?

Proper laboratory use of pipettes includes regular maintenance and cleaning of pipettes. Pipettes can be disassembled and cleaned, and most parts sterilized according to the manufacturer's instructions by autoclaving. This prevents possible contamination of the pipetted liquids, contamination of samples, and deterioration of work. There are three types of contamination during pipetting:

1 **Pipette to sample contamination** – contaminated pipette or tips can contaminate the sample. The best way to avoid this type of contamination is to use filter tips, change the tip after pipetting each sample and clean the pipette regularly.
2 **Sample-to-pipette contamination** – aerosols from samples can enter the cone of the pipette. To avoid aerosol contamination, use filter tips, release the plunge slowly, and keep the pipette vertical when pipetting to prevent liquid from running into the pipette cone.
3 **Sample-to-sample contamination (carry-over)** – the remains of the samples can mix inside the tip and contaminate the next sample which may cause false test results. If you think your pipette is contaminated, clean the pipette properly and autoclave it if needed. The best practice is to change tips between each sample.

3.3.7 How to pipette precisely?

The pipette angle is important for the pipetting and proper functioning of the pipette. The pipette should be always in the vertical position when in use, and in the same position when placed in a holder. If you prefer to create an angle between the source or the target tube and the pipette tip, it is recommended to position in a horizontal direction the tube, not the pipette. Storing the pipette in a vertical position in a suitable holder is also preventing contamination and damage to the pipette. Also,

if you must place the pipette in a horizontal position, do it just on the clean laboratory table, but only if the tip fitted to the pipette is empty or if the pipette is without a tip. Pipette accuracy is calibrated and tested by the manufacturer and each pipette is supplied with a professional certificate. Pipettes, like most of the tools in research, can break down with daily use, resulting in pipetting the wrong volume. Therefore, pipettes must be checked regularly. Technique and variability in pipetting can contribute to a biased analysis of the results. An easy test for checking the pipetting ability of a beginner is repeated pipetting and subsequent weighing (on an analytical scale) of the pipetted volume of water and calculating the coefficient of variation of pipetting (100 µL of water = 100 mg). The variability of pipetting is inversely proportional to the volume of the pipetted liquid and therefore, especially when pipetting liquids with very small volumes, it is necessary to work particularly precisely. Fluid properties also affect the accuracy of pipetting. Adding very thick, viscous, or volatile liquids is challenging and requires caution and considerable experience. When pipetting highly foaming liquids, it is necessary to ensure the absence of air bubbles in the added volume, otherwise, the fluid volume will be inaccurate. The temperature of liquids and the environment is an underestimated parameter that also affects the variability of the pipetted volume. When pipetting solutions with different temperatures, before pipetting, the temperature of the solutions must be approximately settled. A change in the ambient temperature and changes in the air pressure also affects the variability of the pipetted volume with automatic pipettes, therefore it is ideal to ensure a constant temperature of the environment in the laboratory.

4 Searching in medical databases (Alexandra Gaál Kovalčíková)

Before any scientific work in the laboratory, the aim of the research has to be defined and the whole experiment or study has to be correctly planned. Failing to plan correctly or to perform research that has already been done may lead to incorrect interpretation of the results, failure to publish, and spending of public funds in vain. Therefore, any research activity should be preceded by a thorough search in public biomedical databases. There are several possibilities, of which the most common is the National Library of Medicine and its PubMed database, Google Scholar, Web of Science, or Scopus databases. While Scopus and Web of Science are paid databases and under normal circumstances one can access these only with individual or institutional subscription, Google Scholar and PubMed are free and easily accessed. Out of these two, Google Scholar tends to return more results of which many might be irrelevant to Your research. On the other hand, it is simply easy to orient and with some of the most important advanced search features such as date filtering and keywords to include/not to include in the search. However, PubMed is currently the gold standard in biomedical electronic research, and thus this section will focus on tips on how to perform a search using the PubMed database.

The first step is to open any web browser and in the google search bar type “pubmed”. The first returned result by the google search is the National Library of Medicine PubMed search tool PubMed. The second option to open PubMed is simply to type “www.pubmed.gov” into the web browser address bar. Either way will lead you to a web page with one search bar in the middle of the screen, where you can type keywords, and upon hitting the “Search” button next to the search bar will return all results related to the keywords. The initial search engine is designed to include all fields and the return result might read several million. The trick for the search is thus to correctly identify the keywords and narrow the initial search using truncation or using the so-called Boolean operators “AND”, “OR”, “NOT”. Let us explain these in detail.

The truncation option is easy to understand and can be used if we would like to search for all the words having the same word base. For example, if we search for therapy for a certain disease, this can be referred to in the articles as therapy, therapies, therapeutics, therapeutically, therapeutics, etc… Instead of individual search for each word, in this case, using truncation is an option, where we type the base of the word, i.e., “therap” with subsequent * (therap*) into the search tab. This would include searching for all therapeutic approaches or words containing “therap” and would be time-saving.

In general, operators should be capitalized, otherwise, PubMed will not consider these as operators. Let us imagine, You want to search for articles related to cancer in the liver. Typing “liver cancer” into PubMed will return articles related to the liver, cancer, or both. While You are interested in only liver cancer, You should use AND operator, thus narrowing the search for liver cancer only, or texts obtaining both words “liver” as well as “cancer”. The AND operator is thus narrowing Your search in general (Fig. 4).

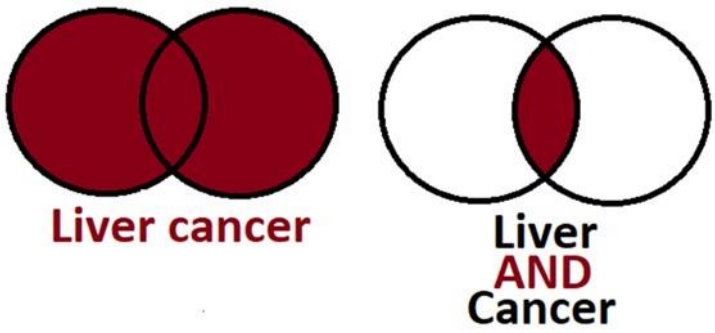


Fig. 4 Keywords and the returned results for PubMed search without and with the Boolean operator AND.

Another example might be that You are looking for articles related to lower limb injury and more specifically You are interested in ankle and foot. The approach might include a search with the key words “trauma” and “ankle”, afterward, You save Your findings, and subsequently do a second search for “trauma” and “foot” and again You save Your search. However, You can use the Boolean operator OR for combined search “trauma” and “(Foot OR Ankle)”. The parentheses ( ) in this case inform the search engine to first search for foot or ankle and then search for trauma in the results where foot or ankle is included. Usually, the OR operator widens the result (Fig. 5).

Fig. 5 Keywords and the returned results for PubMed search with OR operator.

The last operator in the search string can be the NOT operator. NOT operator serves to exclude the word from the search. Imagine You search for surgery repair possibilities for the knee. The knee

consists of three bones as femur, patella, and tibia. Under normal circumstances using the word knee would include surgical repair possibilities for all three bones. However, if You are specifically not interested in one of the bones, for example, patella, You can exclude this from the search. Thus, Your string would be “knee NOT patella”, which would narrow the search more specifically (Fig. 6).

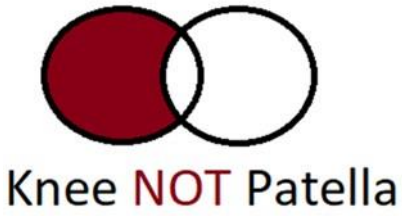


Fig. 6 Keywords and the returned results for PubMed search with the NOT operator.

Indeed, using Boolean operators You can perform more complex searches at once. Let us take the last NOT example, where You would not search for surgery repair, but again for the injury. Now, as in other languages, also in English, there might be several synonyms for one word. The word “injury” has for example the synonymic word “trauma”. Thus, You can search using OR and AND operators to see all articles. The search string would look like “(trauma OR injury) AND (knee NOT patella)”. The parentheses ( ) in this case once again group relevant keywords together, i.e. the search engine searches for trauma or injury, and once found, it searches the text where it is mentioned also knee but without patella. Although the logic behind operators is quite straightforward, it needs little practice with PubMed to fully acknowledge the possibilities. New PubMed design, however, helps even those not acknowledged with the operators to program its string. On the intro page www.pubmed.gov just below the search tab is the “Advanced” button (Fig. 7A). Similarly, if You perform a first search then on the result page You have the “Advanced” search button also just below the search tab (Fig. 7B). The “Advanced” search tab (Fig. 7C) opens, and You can type Your keywords and use corresponding Boolean operators. Hitting the ENTER or ADD button will add Your search to the query box with parentheses, keywords and corresponding operators.

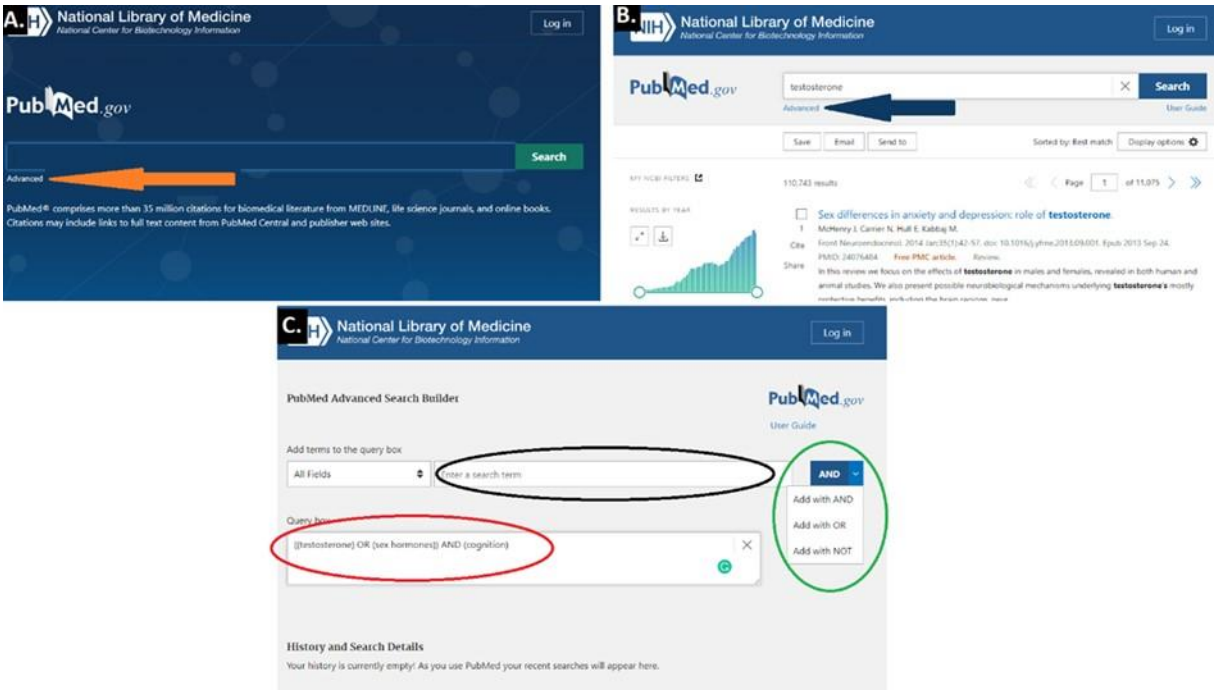


Fig. 7 Advanced search in PubMed. A. Location of Advanced button on PubMed homepage (orange arrow) and B. after initial search was performed (blue arrow). C. is the Advanced search tab, where the black circle points towards the search tab, the green circle chooses Boolean operators AND, OR, NOT and the red oval demonstrates the query box with the search string.

Lastly, as mentioned at the beginning of this chapter, simply typing the keywords into the search tab might not be sufficient, since the search engine returns any articles that do contain in the available text either of the words entered. This might not be ideal if these words are commonly used, or if the search engine returns many articles containing our keywords but only as a secondary outcome, not as a major topic of interest. Let's use the above example, where we used the keywords testosterone, sex hormones, and cognition. While we still need operators for testosterone and sex hormones, we want to narrow the search, so the keywords are included directly in the title of the article. Moreover, we want all papers which are on this topic and where Hodosy is the author. The click on the "All Fields" button drops the menu, where we choose Title and in the search tab, we type testosterone. After hitting ENTER, the string testosterone [Title] is added. Similarly, we continue with sex hormones in the search tab, just we change AND operator into the OR operator and hit ENTER. The string then would look as "(testosterone [Title] OR sex hormones [Title]). Finally, we change the field to Author and type Hodosy into the search tab. We change the operator into AND and hit ENTER. Final search string would look like "((testosterone [Title]) OR (sex hormones [Title])) AND (hodosy[Author])". Once ready, we can hit the Search button and we will get the result, where Hodosy is the author of

articles that have testosterone or sex hormones in the title. While the round brackets are used to group keywords together, square brackets [ ] are used to define the field of the keywords (Fig. 8).

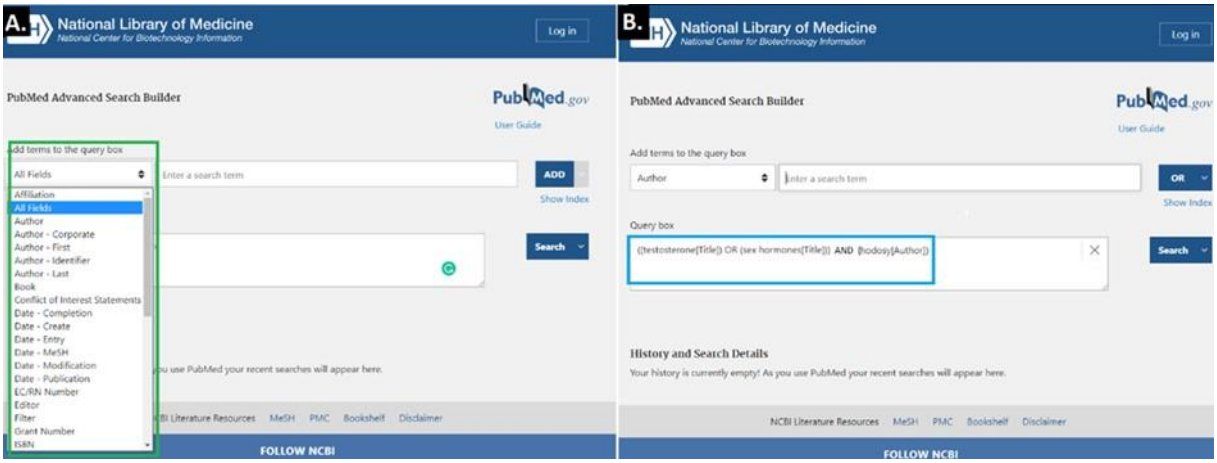


Fig. 8 Advanced search and using fields to narrow the search. A. Universally, All fields are selected (Green box), but as can be seen, many other fields such as year/date, author, title, and many others can be selected for the keywords. B. The query box then changes correspondingly (blue box). Note that round parentheses are used for grouping the keywords, while square brackets are used to define the field of the keyword.

Wise use of operators and truncation and correct keywords can narrow the search very much and return relevant articles that You are interested in. Nevertheless, Your aim for the research might be different. For example, scientists for basic research prefer searching databases for animal studies, while a clinician wants to see what has been already tried on humans. Also, You might be interested in certain recent studies, not the old ones (or vice versa). As a clinician, You might do Your research based on strong evidence, or on contrary, You search for case studies and whether there are any. None of the operators, truncation, or keywords can help You return the relevant articles from this point of view. Or this might be possible, but the search string would be too complicated. Therefore, we prefer a simple search string and once You have got the first results, the result screen has additional filters on the left side of the returned articles. Applying these filters can help You to find what You need (Fig. 9).

In conclusion, as for any job performed, it is necessary to practice and experiment with the operators, filters, and keywords. Although You do not have to create an account, after account creation, which is free, PubMed offers many other advanced features and personalized settings that can help You find Your topic much faster, You may set up alerts for what You are interested in, You can create citations

export into the clipboard and much more. Do not worry to try and experiment with these. Also, there are several tutorials available regarding Pubmed Search or Advanced Pubmed Search that might be helpful.

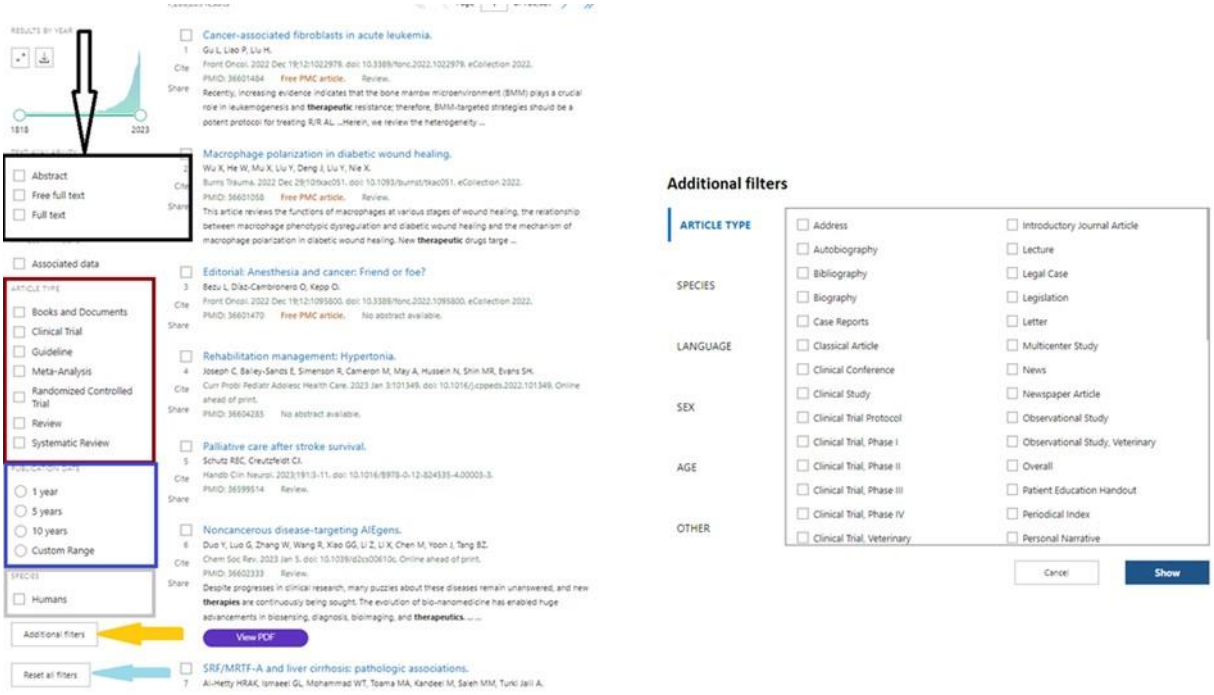


Fig. 9 After a search, the left panel (black arrow) shows additional filters. The most commonly used filters include Text availability (black box), Article type (red box), Publication date (blue box), and Species (grey box). If these filters are not enough, hitting Additional filters (orange arrow) will pop up an additional screen (right side of the pictures) with more filter options. Tip: If after typing keywords You do not get any results, try to click Reset all filters (light blue arrow) to reset previous filters, which You might forget to turn off.

5 Basic calculation of the coefficient of variation (Katarína Šebeková)

The coefficient of variation (CV) is a measure of imprecision of the (chemical) assay or any analysis (e.g., morphometric measurement) that produces continuous values. The CV reflects the performance of the assay ***in the hands of the user*** (e.g., depends upon the test methodology, the instrument being used, the precision of pipetting (data acquisition), measurement, recording (if data are transcribed manually), and the range of results).

The CV shows the extent of variability in relation to the mean, e.g., it indicates the variability of the test results. It is calculated as the ratio of the standard deviation to the mean, with the result generally reported as a percentage (% CV, formula 1):

CV (%) = Standard Deviation / Mean × 100

The higher the CV, the greater the dispersion. CV is dimensionless (i.e., independent of the unit in which the measurement was taken) and thus, comparable between data sets with different units or widely different means.

To express the precision, or repeatability, of biochemical test results, two measures of the CV are generally reported: The Intra-Assay CV (calculated from the mean values for the high and low concentrations on the plate) and the Inter-Assay CV (plate-to-plate consistency, each plate is run with its own calibration curve, again indicated for low and high concentrations).

- **CVs should be calculated from the calculated concentrations** rather than the raw optical densities.
- The CVs should be reported for **concentrations that reflect the physiological and pathological range** in each biological material analyzed, as concentrations in different body fluids (saliva, urine, plasma/serum, cerebrospinal fluid) might differ.
- **Acceptability of CVs** depends on the method; CV is generally lower for intra-assay than inter-assay measurement, lower for the higher than lower concentrations of the analyte, lower for simple spectrophotometric and fluorometric assays than ELISA assays.
- **Spectrophotometric, fluorimetric, ELISA methods** – **general:** probes for the calibration curve including blanks as well as unknown samples are to be pipetted in duplicates, unless not indicated differently by the manufacturer or in the Standard Operating Procedures (SOPs) at IMBM (e.g., in triplicates).
- **CVs for e.g., spectrophotometric and fluorometric methods** should be reported for the low and high concentration of the analyte as to intra-assay (generally 10 replicates/sample) and

inter-assay (generally as day-to-day variability, e.g., running the same samples on five consecutive days, based on daily calibration curve).

- **ELISA methods:** generally, **intra-assay** CVs are reported based on means from the duplicates of analyzed samples.

Remark: The reproducibility of the assay is to be controlled:

- By checking the absorbances of the standards and the blank across the calibration curve (comparison with former data obtained from the same reader employing the same method and calibration)
- If controls for upper and lower range values are included in the kit: by checking whether their concentrations are within the indicated interval indicated by the manufacturer.
- If the controls are not provided by the manufacturer: it is advisable using internal controls, e.g., pooled sample with known mean and SD.
- As to the urinary protein concentrations using the pyrogallol red method employed to assess the performance of students at IMBM (training the pipetting accuracy): the intra-assay CV: lower range of the calibration curve: less than 8 %; for concentrations within the upper range of the calibration curve CV about 3 % is generally acceptable.

Procedure:

1. Construct a template (Tab. 2).

2. Pipet the standards, blanks (in duplicates), and biological samples (ten plicates each) according to the protocol, run the reaction.

3. Perform the readouts to obtain raw data (Tab 3).

4. From the raw absorbance data subtract the mean blank value (Tab. 4).

5. Construct the calibration curve (e.g., formula 2: $y = ax + c$) from means of background corrected mean absorbances versus concentrations of the standards (Tab. 5 and Fig. 10).

- Calculate R^2 to evaluate the accuracy of the fit.

6. Calculate the concentrations of all biological samples using the formula 1 derived equation (e.g., $x = (y - c) / a$).

- If just training the pipetting and checking your CVs, calculate also the concentrations of each standard.

7. Calculate the CVs (for each of the 8 unknown samples pipetted in 10 plicates, Tab. 6); and standards pipetted in duplicates if training, Tab. 7).

Example: advanced oxidation protein products in plasma – calibration curve and CV of 8 different rat plasma samples: performed on 19th February 2003; technician: ZS.

Tab. 2 TEMPLATE: S1 – S5 – standards, BL – blank, T1 – T8 – test samples.

Standards	Concentration
S1	100 μmol/l
S2	70 μmol/l
S3	50 μmol/l
S4	30 μmol/l
S5	10 μmol/l

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	S1	S1	T1	T1	T1	T1	T1	T1	T1	T1	T1	T1
B	S2	S2	T2	T2	T2	T2	T2	T2	T2	T2	T2	T2
C	S3	S3	T3	T3	T3	T3	T3	T3	T3	T3	T3	T3
D	S4	S4	T4	T4	T4	T4	T4	T4	T4	T4	T4	T4
E	S5	S5	T5	T5	T5	T5	T5	T5	T5	T5	T5	T5
F	BL	BL	T6	T6	T6	T6	T6	T6	T6	T6	T6	T6
G			T7	T7	T7	T7	T7	T7	T7	T7	T7	T7
H			T8	T8	T8	T8	T8	T8	T8	T8	T8	T8

Tab. 3 Raw data: absorbance.

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.907	0.877	0.591	0.58	0.603	0.612	0.588	0.564	0.6	0.596	0.604	0.62
B	0.689	0.692	0.402	0.42	0.433	0.427	0.426	0.433	0.44	0.405	0.43	0.427
C	0.548	0.516	0.155	0.16	0.172	0.174	0.169	0.158	0.159	0.163	0.165	0.17
D	0.356	0.358	0.359	0.36	0.375	0.355	0.359	0.362	0.355	0.371	0.364	0.363
E	0.163	0.175	0.822	0.84	0.847	0.837	0.822	0.844	0.835	0.834	0.828	0.832
F	0.08	0.079	0.18	0.189	0.195	0.193	0.185	0.187	0.192	0.186	0.188	0.198
G			0.126	0.13	0.135	0.128	0.136	0.14	0.146	0.127	0.136	0.144
H			0.769	0.778	0.724	0.756	0.761	0.768	0.78	0.784	0.777	0.781

Blank mean (F1-F2): 0.0795

Tab. 4 Raw data minus background (mean blank).

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.8275	0.7975	0.512	0.501	0.524	0.533	0.509	0.485	0.521	0.517	0.525	0.541
B	0.6095	0.6125	0.323	0.341	0.354	0.348	0.347	0.354	0.361	0.326	0.351	0.348
C	0.4685	0.4365	0.076	0.081	0.093	0.095	0.090	0.079	0.080	0.084	0.086	0.091
D	0.2765	0.2785	0.280	0.281	0.296	0.276	0.280	0.283	0.276	0.292	0.285	0.284
E	0.0835	0.0955	0.743	0.761	0.768	0.758	0.743	0.765	0.756	0.755	0.749	0.753
F	0.0005	-0.0005	0.101	0.110	0.116	0.114	0.106	0.108	0.113	0.107	0.109	0.119
G			0.047	0.051	0.056	0.049	0.057	0.061	0.067	0.048	0.057	0.065
H			0.690	0.699	0.645	0.677	0.682	0.689	0.701	0.705	0.698	0.702

Tab. 5 Calibration curve construction.

Standards	Concentration (µmol/l)	Mean absorbance
S1	100	0.813
S2	70	0.611
S3	50	0.453
S4	30	0.278
S5	10	0.090
BLANK	0	0.000

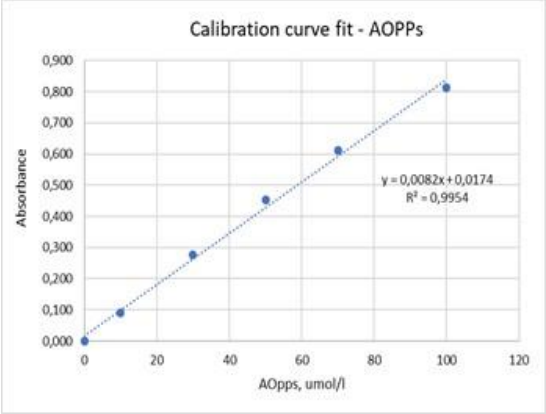


Fig. 10 Calibration curve – an example.

The calibration curve should consist of **five to eight points that cover the entire range of expected (linear) analyte concentrations in the test samples.**

R^2 of the calibration curve: The coefficient of determination (R^2 value), is **a measure of how well a set of data fits a calibration curve**. A good standard curve should have an R^2 value **as close to 1 as possible but ≥ 0.95**. Generally, **values** of **R^2** < 0.95 are not acceptable as it means that your **calibration** by itself yields a 5% variability error in your measurement.

Tab. 6 Calculation of test samples AOPPs concentration: x=(y-0,0174)/0,0082 and the CV of T1-to T8 samples.

	1	2	3	4	5	6	7	8	9	10	11	12	mean conc.	SD	CV (%)
A			60.3	58.9	61.7	62.8	59.9	57.0	61.4	60.9	61.8	63.8	60.8	2.0	**3.2**
B			37.2	39.4	41.0	40.3	40.1	41.0	41.8	37.6	40.6	40.3	39.9	1.5	**3.7**
C			7.1	7.7	9.2	9.4	8.8	7.5	7.6	8.1	8.3	8.9	8.2	0.8	**9.6**
D			32.0	32.1	33.9	31.5	32.0	32.3	31.5	33.4	32.6	32.5	32.4	0.8	**2.4**
E			88.4	90.6	91.5	90.3	88.4	91.1	90.0	89.9	89.2	89.6	89.9	1.0	**1.1**
F			10.1	11.2	12.0	11.7	10.7	11.0	11.6	10.9	11.1	12.3	11.3	0.6	**5.7**
G			3.5	4.0	4.6	3.8	4.8	5.3	6.0	3.7	4.8	5.7	4.6	0.9	**18.6**
H			82.0	83.1	76.5	80.4	81.0	81.8	83.3	83.8	82.9	83.4	81.8	2.2	**2.7**

Interpretation: it is expected that the CVs of samples with AOPPs concentration above 30 µmol/l will range below 5%; those with concentrations ranging from 10 to 30 µmol/l below 10 %. and those with concentrations lower than 10 µmol/l would reach CVs below 15 %.

Outcome: for the samples in the higher AOPPs range (T1, T2, T4, T5, T8), as well as those in the range of about 10 µmol/l (T3 and T6) acceptable CV, was reached. CV of the sample T7 is higher than expected. The performance of the technician for this concentration of the analyte is to be checked based on the frequency of biological samples with such low concentrations of AOPPs. If the frequency of the samples with such low concentrations is very rare. CV below 20 % at as low a concentration as it is, is still acceptable.

Remark: CVs are always to be interpreted considering the positioning of the particular point of the calibration curve at the computed regression line.

This is an example of intra-assay variability. The inter-assay analysis is generally done on 5 consecutive days – the same samples, pipetted in duplicates, always with the standard curve on the

plate. Acceptability of CVs varies according to the method of analysis and biological material (e.g., acceptability of CV might differ between biological fluids analyzed for the same analyte).

Tab. 7 Calculation of CV of the standards

	Concentration 1	Concentration 2	Mean conc.	SD	CV
A	**98.8**	**95.1**	**97.0**	**2.59**	**2.7**
B	**72.2**	**72.6**	**72.4**	**0.26**	**0.4**
C	**55.0**	**51.1**	**53.1**	**2.76**	**5.2**
D	**31.6**	**31.8**	**31.7**	**0.17**	**0.5**
E	**8.1**	**9.5**	**8.8**	**1.03**	**11.8**

Interpretation: It would be expected that CVs rise with a declining concentration of standards. The higher CV than expected according to obtained results (rows A and C – theoretically 100 and 50 µmol/l) probably reflects the imprecision in the pipetting, but they are still acceptable as these points do not represent major outliers as follows from the calibration curve. CV of 11. 8% for the theoretical concentration of 10 µmol/l is acceptable.

6 Design of a study or experiment and project summary (Nadja Šupčíková)

Before starting a study, you need to create a research plan that will help you to describe what will you investigate, why it is important and how will you conduct your research. The format can be different depending on what is the design of study intended for (grant application, part of school application, etc). In either case, you should carefully consider all sections of research plan. Besides helping you defining your research, clear research plan can be used to show a sponsor, academic institution, or potential supervisor that your idea is meaningful and worthwhile. Prior to performing a design study, a thorough review of literature is necessary.

1. **Research title, authors, co-authors, and supervisor**

Every research needs a title that should reflect the main point of the research itself. In many cases, there is also acronym, which is a shortened title used in everyday communication or for easier and faster recognition between different research projects when there are more of them at one institution. It is also necessary to state your name and names of your co-authors as well as the supervisor.

Example: Let's say that we are preparing a research plan for a study that will be focused on neutrophils role in the pathogenesis of urinary tract infection. We are planning to do an animal model in which we will use a specific bacteria strain – so called uropathogenic *Escherichia coli*. So, the possible title of the project could be Neutrophils in urinary tract infection caused by uropathogenic *Escherichia coli*. Acronym for such a study could be NEUTI as a shortcut referring to both neutrophils (NEU) and urinary tract infection (UTI).

2. **Consider your aims and approach**

You should already have a clear understanding of the research subject you wish to explore before you begin designing your study. There are several approaches you may take to answer this subject. Your goals and priorities should impact your study design decisions; start by carefully considering your goals.

Your initial decision should be to choose between a qualitative and a quantitative approach. Qualitative research designs are frequently more adaptable, enabling you to change your strategy in response to what you discover during the research process. Quantitative research designs are typically more fixed, which means that variables and hypotheses are usually well established prior to data collection. Another option is to apply a mixed-methods design that combines features of both and get a

more comprehensive understanding of the issue under investigation and to increase the validity of your conclusions.

Example: In NEUTI study we would go with the quantitative research design. In this case a hypothesis will be formulated based on the literature research. A hypothesis is statement of expectation or prediction that will be tested by research. Several quantitative parameters will be used that will either confirm or disprove hypothesis.

3. Choose a type of research design

There are many different study design options available for both qualitative and quantitative methodologies. Each type offers a framework for your research's overall design.

Quantitative research designs

Experimental design:

- used to evaluate causal relationships,
- involves changing an independent variable and measuring its effect on a dependent variable,
- subjects are randomly assigned to groups,
- usually conducted in a controlled environment (e.g., a lab).

Quasi-experimental design:

- used to evaluate causal relationships,
- without random assignment,
- typically includes comparing the results of existing groups,
- usually conducted in a natural environment.

Correlational design:

- used to examine the existence and strength of a relationship between two variables,
- variables are not influenced while being measured.

Descriptive design:

- used to characterize attributes, averages, trends, etc.,
- variables are not influenced while being measured.

Qualitative research designs:

Case study:

- detailed study of a specific subject (e.g., a place, event, organization, etc.),
- data can be gathered from a wide range of sources and techniques,
- emphasizes getting a comprehensive grasp of the situation.

Ethnography:

- detailed study of the culture of a specific community or group,
- data is collected by extended immersion and close observation,
- focuses on describing and interpreting beliefs, conventions, social dynamics, etc.

Grounded theory:

- aims to create a theory by methodically analysing qualitative data.

Phenomenology:

- uses participant perspectives to describe a phenomenon or occurrence to understand it.

Example: In NEUTI study we will use experimental design because we will change independent variable (animal strain with and without specific gene important for neutrophils to form neutrophil extracellular traps) and observe the effect on a dependent variable (how many neutrophils form neutrophil extracellular traps).

4. Identify your population

Your research design should exactly specify who or what will be the subjects of your study and how participants or subjects will be chosen. Any clinical research should have clear inclusion and exclusion criteria defined. While it would be best to include whole population in the study, this is often unrealistic and not possible. Therefore, in research, a sample from the population is used. The sample is defined as smaller but representative group of people you'll collect data from. On the other hand, a population is the overall group you wish to make conclusions about. Whatever you want to investigate can be included in a population, including plants, animals, groups, texts, nations, etc. The more precisely you define your population, the easier it will be to gather a representative sample.

Example: In the NEUTI study, both males and females of the given mouse strains will be included in order to describe the role of netosis in both sexes.

5. Data collection

Methods for collecting data are strategies to systematically measure variables and collect data. They provide you the chance to learn about your study issue directly and to develop original ideas. You can use only one data collection technique or a combination of methods in the same study.

Survey methods

Through surveys, you can directly question people about their thoughts, attitudes, experiences, and qualities. The two primary survey techniques are questionnaires and interviews.

Observation methods

With observational research, you can gather data anonymously by observing characteristics, behaviours, or social interactions rather than relying on self-reporting. You can perform observations in real time while taking notes, or you can record your observations on audio and/or video for subsequent study. These methods also include measurement of parameters determined in advance.

Example: In a type of study such as NEUTI, only observation methods can be used.

6. Plan data assessment

You must choose your methods and then carefully determine how you will apply them to get data that is reliable, accurate, and unbiased. Planning systematic methods is crucial in quantitative research since it requires precise variable definition and good measurement validity and reliability.

Sampling procedure – it is important to plan your research to make decisions on question such as *How many participants are necessary for a sufficient sample size?* Or *What criteria for inclusion and exclusion will you use to determine who is eligible to participate?* You should also consider time needed for realization of data collection.

Example: In animal studies you must carefully consider what and when will be done with animals. So, if we want to use an UTI animal model, we must decide if we want to determine role of neutrophils in acute infection (24 hours) or infection developing to upper urinary tract (7 days or 14 days). We also must consider duration of all methods, for example operation of one animal can last up to 30 minutes or determining colony forming units in any fluid takes at least 1 day.

7. Data analysis

Your research question cannot be answered by raw data alone. The preparation of your data analysis strategy is the final step in designing your research. With the help of descriptive statistics, you may reduce your sample data into the following:

• data's distribution (for example, the frequency of each test result),

• data's central tendency; (e.g., the mean to describe the average score),

• variability of data (e.g., the standard deviation to describe how spread out the scores are).

You can test hypotheses about a link between variables and make estimates about the population based on your sample data by using inferential statistics. While comparison tests, like t-tests and ANOVAs, look for differences in the results of several groups, regression and correlation tests look for relationships between two or more variables. Several elements of your research design, such as the types of variables you're working with and the distribution of your data, will influence the statistical test you choose.

Example: If we will compare only the SHAM (the bacterial culture was exchanged for control fluid) and UTI group of the same animal strain we can use t-test for comparison. If we want to determine the effect of both procedure (SHAM vs UTI) and animal strain, we have to use ANOVA.

Name:	project title
Responsibility:	responsible person and supervisor
Co-authors:	all team members who will participate in project
Acronym:	shortened name for easier project reference
Aim of the project:	study question/s
Hypotheses:	specific assumptions about the relationships between study variables, which can be statistically tested
Sample:	type of samples (e.g. saliva, urine, stool, etc)
Sex:	define sex of your study individuals
Age:	define age of your study individuals
Number:	define number of whole study individuals or per each group
Groups:	define types of groups (e.g. disease group and control group)
Timeline:	estimate duration of each step of the study (e.g. 1st week - specimen measurement, 2nd week - data analysis)
Main readouts:	define parameters important for the test of hypothesis
Secondary readouts:	define parameters that are not important for the test of hypothesis but will be collected during research
List of methods:	define all methods, which will be used in research (e.g. specimen collection, protein measurement, etc.)
Key publications:	list 1-2 publications that are relevant to the topic
Estimated duration of the project:	estimate overall duration of the project
Notes:	add any notes that are important during conducting of the research (e.g. normalization of data to creatinine in case of urine samples)

Fig. 11 Example of research plan – Project summary.

Name:	Neutrophils in urinary tract infection caused by uropathogenic *Escherichia coli*
Responsibility:	NŠ
Co-authors:	ĽT
Acronym:	NEUTI
Aim of the project:	Neutrophils of PAD4 deficient mice supposedly shouldn't be able to form neutrophil extracellular traps and thus they should have aggravated pathogenesis of UTI
Hypotheses:	We expect higher microbic activity and lower netosis in PAD4 deficient mice in comparison with wild-type mice.
Sample:	blood, urine, bladder, kidney
Sex:	both female and male
Age:	2-3 months
Number:	male/female
Groups:	4 - WT SHAM, WT UTI, KO SHAM, KO UTI
Timeline:	1. week - UTI induction, blood/urine collection (24h, 3d), flow cytometry, SP-SDS 2. week - sacrifice, organ harvest, blood/urine coll. (7d), flow cytometry, SP-SDS 3. week - data analysis, ppt with results, writing of final report
Main readouts:	formation of NETs
Secondary readouts:	CFU, histology
List of methods:	cultivation of bacteria, LB broth prep., anaesthesia, mini surgical model, SP-SDS, CFU determination, flow cytometry, dissection, organ harvest, organ homogenization, data processing
Key publications:	Tsourouktsoglou TD, Warnatsch A, Ioannou M, Hoving D, Wang Q, Papayannopoulos V. Histones, DNA, and Citrullination Promote Neutrophil Extracellular Trap Inflammation by Regulating the Localization and Activation of TLR4. Cell Rep. 2020;31(5):107602.
Estimated duration of the project:	3 weeks
Notes:	

Fig. 12 Example of research plan – Project summary 2.

Name:	Modeling of autistic disorders - Home cage monitoring of Shank3 mice
Responsibility:	MR, JS
Co-authors:	PH, ER, PC
Acronym:	HOME-SHANK3
Aim of the project:	To investigate behavioral profile of Shank3 mice during 24 hours home-cage monitoring
Hypotheses:	Shank3 deficient mice will exhibit hypoactivity during active phase and distruption of sleep activity during inactive phase
Sample:	Mice
Sex:	Male/Female
Age:	Adult
Number:	
Groups:	4 - Shank3 male, Shank3 female, WT male, WT female
Timeline:	1. week- learning of methodology, research of publications, setting up the design 2. week- testing, statistical evalutation, preparation of the output
Main readouts:	home-cage monitoring
Secondary readouts:	sleep deprivation, hypoactivity
List of methods:	Home cage monitoring with Phenotyper camera (24h), Ethovision XT software
Key publications:	C. C., Tudor, J. C., Ferri, S. L., Jongens, T. A., & Abel, T. (2019). Home-cage hypoactivity in mouse genetic models of autism spectrum disorder. Neurobiology of learning and memory, 165, 107000. https://doi.org/10.1016/j.nlm.2019.02.010
Estimated duration of the project:	2-3 weeks
Notes:	

Fig. 13 Example of research plan – Project summary 3.

Name:	Does prenatal and postnatal cafeteria diet lead to chronic inflammation in rat offspring???
Responsibility:	AF, KŠ
Co-authors:	VB, VŠ, LM
Acronym:	PreCaf, PostCaf
Aim of the project:	The project aims to investigate the effect of both prenatal and postnatal cafeteria diet consumption on development of chronic inflammation in peripheral tissues (adipose tissue, muscles) and liver of rat offspring.
Hypotheses:	Pre/Postnatal cafeteria diet consumption leads to MetS with chronic infammation of peripheral tissues.
Sample:	Wistar rats
Sex:	female, male
Age:	from PND 0 up to 4 months of age
Number:	52 (10, 10, 10, 10, 6, 6)
Groups:	CTRL females, CTRL males, FFD females, FFD males, CAF females, CAF males
Timeline:	caffeteria diet/ McDonald diet administration-14 weeks, 2 weeks analysis - at the end of each month of age MetS and inflammatory parameters
Main readouts:	Metabolic: insulin resistance (fasting glucose) dynamics (monthly), OGTT dynamics (monthly), blood pressure, DEXA, morphometry; HDL, LDL, TAG Inflammation: ecDNA- dynamics (monthly), Nethosis- dynamics (montlhy), TNF alfa, IL-6
Secondary readouts:	histology and imunohistochemistry of adipose tissue, TAG/CHOL-liver
List of methods:	OGTT, blood collection, blood pressure measurement, DEXA, isolation of ecDNA, biolis, ELISA, netosis
Key publications:	Matuszewska, J., Zalewski, T., Klimaszyk, A. et al. Mothers' cafeteria diet induced sex-specific changes in fat content, metabolic profiles, and inflammation outcomes in rat offspring. Sci Rep 11, 18573 (2021). https://doi.org/10.1038/s41598-021-97487-x
Estimated duration of the project:	4 months
Notes:	

Fig. 14 Example of research plan – Project summary 4.

7 Work with laboratory animals (Veronika Borbélyová)

7.1 Basic physiological and reproductive data of laboratory rats and mice

Laboratory rats and mice are the preferred species used in biomedical research because these animals have many similarities to humans in terms of anatomy, physiology, and genetics. Besides that, the use of laboratory rats and mice for research purposes has some advantages including small size, ease of maintenance, and short life cycle. Laboratory rats and mice have short gestation with a relatively large number of offspring, with rapid development to adulthood and a relatively short life span. These properties of laboratory rats and mice make them suitable for relatively rapid experimental studies. For successful work with laboratory rats and mice, it is essential to know some basic information about their reproductive biology (Tab. 8) and physiology (Tab. 9), which we have summarized in Tabs.

Tab. 8 Basic reproductive data of laboratory rats and mice.

	Rats	**Mice**
Weight of pups at birth:	5-6 g	1 g
Weight of the pups at weaning:	40-50 g	10-15 g
Body weight of adult animals:	Males: 300-500 g Females: 250-300 g	Males: 20-40 g Females: 25-40 g
Age at weaning:	21 days	25-28 days
Puberty:	at age of 6-7 weeks	at age of 5 weeks
Full sexual maturity:	at age of 8-10 weeks	at age of 7-9 weeks
Estrous cycle length:	4-5 days	4-5 days
Sexual receptivity during estrus:	12 hours	12 hours
Fertilization:	2 hours after mating	2 hours after mating
Gestation period:	20-23 days	19-21 days
Litter size:	4-15 pups	6-12 pups

Tab. 9 Basic physiological data of laboratory rats and mice.

	Rats	Mice
Life span:	2.5-3.5 years	1-3 years
Heart rate:	250-600 / min	300-800 / min
Respiration rate:	70-150 / min	90-220 / min
Body temperature:	35.8- 37.5 °C	35.5-37.4 °C
Daily water consumption:	10-12 ml / 100g	1.5 ml / 10g
Daily food consumption:	15-30 g	3-6 g
Urine:	clear and yellow	strong odor, clear to yellow
Feces:	firm, dark brown, elongated, round ends	firm, rice-sized, dark brown

Without knowing basic information about reproductive biology such as sexual maturity in laboratory rodents it could happen that we will mate the females with males too early, and it can lead to that a sexually immature female will either abort or give birth to non-viable pups. Another example is, when we are testing the behavior of animals throughout their life span, we have to know when the animals are at pubertal age or when the animals are already in adulthood.

7.2 Handling of laboratory rodents (Veronika Borbélyová)

Handling is defined as manipulating laboratory rodents with your hands either in a direct (we touch the animals with our hands) or indirect way (we do not touch the animals with our hands). Handling itself is a process thanks to which laboratory animals habituate for manipulation with a given experimenter. You have to always handle laboratory rodents very carefully. An appropriate way of handling helps to reduce stress in animals under experimental conditions.

7.2.1 Handling of laboratory mice

Compared to laboratory rats, laboratory mice have no strong drive to cooperate. For that reason, it is important to gain skills in handling procedures of laboratory mice. Correct handling should be implemented not only during the given experiment but should already start at the time of importing animals to the animal house to familiarize animals with the experimenter and also manipulations.

Gaining skills in handling is beneficial not only from the point of view of the experimenter, who with appropriate handling reduces the chance of the mouse biting his fingers but also from the point of view of the mouse which gets familiarized with proper handling procedure and after a certain time handling itself will no longer be a stressful situation for the animal.

Forms of laboratory mouse handling

There are several ways of handling laboratory mice. In general, handling can be restricted to an individual mouse or a group of mice. As laboratory mice tend to rest in a group in one corner of the cage it makes it easier to move a group of animals with your **two hands** to another cage (Fig. 15 A). An indirect way of transferring a laboratory mouse or a group of mice is by using a **glass or synthetic bowl** (Fig. 15 C) or a **paper roll** (Fig. 15 B).

A **short transfer** (less than 2-3 s) of a mouse to another place is also possible by grasping the mouse by the base of the tail, lifted and put to a new location. However, this method of transfer cannot be applied to mice with a higher body weight or to pregnant females In this case, the experimenter should gently guide the animal with one hand to the other hand, while the animal is carefully lifted with holding the base of the tail to avoid its escape.

When transferring a mouse over a **longer distance** the mouse should be placed on the hand and should not be carried by the base of the tail.

Another way of mouse transferring is similar to the **way the mother moves the pups** in her mouth and requires more experience. In this case, the skin of the mouse is gently raised behind the fold of the neck.

In the case of **transferring the mother with her litter**, the mother has to be removed from the cage at first to avoid the stress of the mother and also her defensive behavior as a response to taking out her nest with pups out of the cage. Following mother removal, the whole nest with pups is transferred into another cage, however, if it is possible, without touching the pups. This way of litter transferring is not stressful for the mother, she accepts her pups without any problems in a new environment.

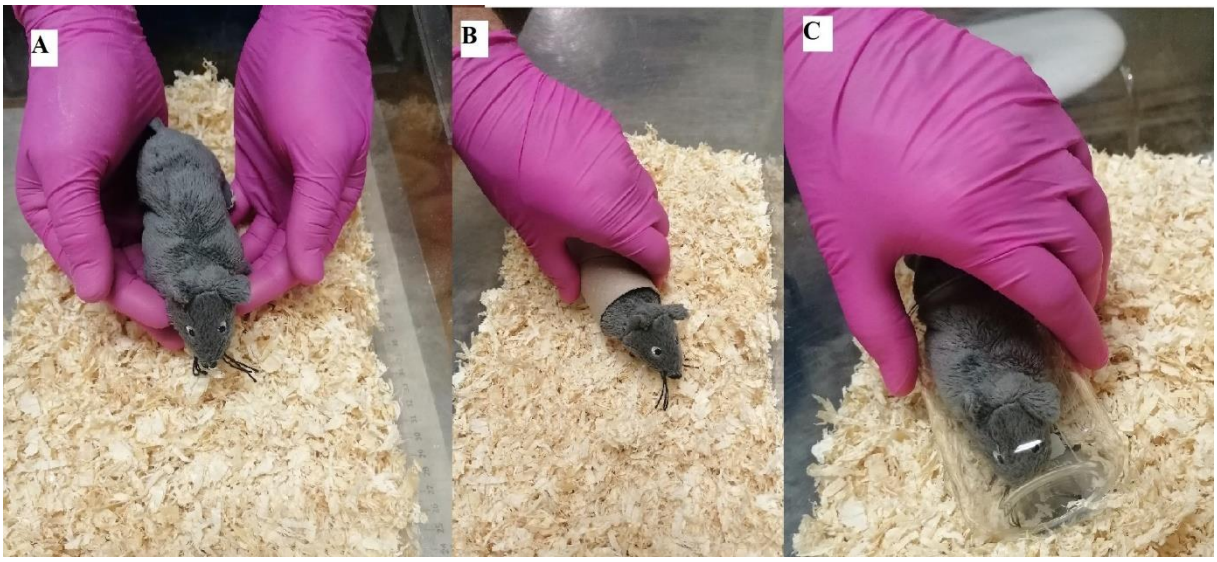


Fig. 15 Types of laboratory mouse transfer. Transfer of the mouse using both hands (A). Transfer of the mouse using a paper roll (B) and a glass baker (C). Photo by Veronika Borbélyová.

7.2.2 Handling of laboratory rats

In contrast to the laboratory mouse, the laboratory rat is a contact animal, and, when handled properly and systematically from early in its life, quickly adapts to individual forms of handling and other procedures. As we have mentioned above, handling means the manipulation with an animal to transfer it to another cage, or for weighing and transferring it to the home cage.

In general, when working with laboratory rats, the experimenter needs to be self-confident, as any hesitation on the side of the handler can lead to stress in the animal, or on the contrary, provoke aggressive behavior.

Handling of adult laboratory rats **by the base of their tail** is permissible when the animals are transferred from one cage to another cage that is close together. This method can also be used when you transfer the animal from the home cage to the balance pan due to weighing as it is quick and it has no harmful effects on rats. However, do not use this method of handling in the case of obese rats, or if you are transferring the rat for a longer distance.

However, if the transfer of adult laboratory rats lasts more than 2-3 s, you have to pick up the animal **with two hands** – at the base of the tail with one hand and immediately put the rat with its abdominal side to another hand or a flat surface.

Neonates should not be held at the base of their tails, as it can lead to injury. For example, young pups (5-6 days old) can be lifted with **both hands**, **or one hand** using all of our fingers to avoid escaping. Once the pups reach 2 weeks of age the method of handling described earlier for adult rats can be employed.

7.3 Marking of laboratory rodents (Veronika Borbélyová)

If you are doing an experiment, you have to know that animals belong to even control or experimental groups. For that reason, you have to mark your animals. You can choose a **permanent or temporary way** of animal marking. While you are marking your animals you are manipulating laboratory rodents with your hands, that is why marking animals is included within the handling process.

Currently, there are several ways of identification of laboratory rodents:

1. **Temporary marking of laboratory animals**:

It can be used for short-term individual identification. It includes:

- marking of an animal's ear or tail with a non-toxic permanent marker to write numbers, lines, or other distinguishable signs,
- marking the fur of the animal mainly on the head or back with marking paints,
- shaving one side or back of an animal's body.

While the advantage of these methods is that they are non-invasive, the disadvantage is that these markings disappear within 1-2 days (marking with permanent marker), 1-2 weeks (paint marking), or 1-4 weeks (shaving of the body). Regarding temporary markings, at IMBM we use mainly the marking of the tail with permanent marker, and we repeat the marking of animals at least twice a week (numbers – tail of rats, lines – tail of mice).

2. **Permanent marking of laboratory animals**:

Different ways of permanent marking of laboratory rodents exist as well:

- implantation of microchips – under the skin between the shoulder blades (needs anesthesia),
- tattooing – on the tail (needs anesthesia),
- micro-tattooing – ink injection to toe pads or ears,
- **ear punching** – commonly used at IMBM,
- **ear tags** – commonly used at IMBM.

Ear punching:

This method cannot be used in rodents under 2 weeks of age, however, you can create your system for animal marking. In the literature we can meet with several systems of ear punching – making holes, the most commonly used is when:

- the right ear is used for one-digit ID numbers,
- the left ear is used for two-digit ID numbers,
- the middle of the ear for three-digit numbers (Fig. 16).

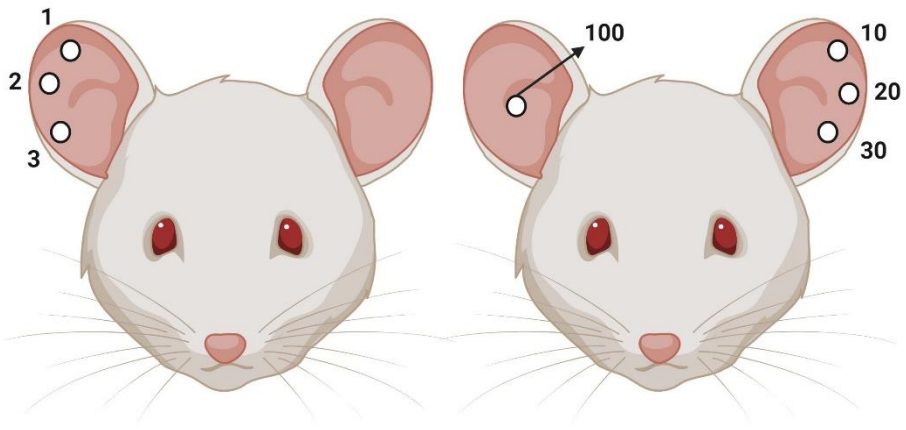


Fig. 16 Ear punching method for marking and identification of laboratory rodents. Created with BioRender.com.

This way of animal marking needs some skills regarding the immobilization of laboratory rodents (we will discuss immobilization methods in the next chapter) as you have to immobilize the animal in a way to have the ears of the animal accessible to make holes using an ear punch. When you are using an ear punch, place it 3 mm from the edge of the ear, make the hole, and gently lift the ear punch. then open and remove it from the ear. Clean the ear punch before and also after ear punching with 70% of ethanol.

You have to pay attention when using this type of marking, as if you make a hole too deep in the ear you can cause injury to the animal. In addition, if you make the hole too close to the edge of the ear, it can very easily tear and you will not know the ID of the animal.

Ear tagging:

Ear tags can be metal or plastic and are easily placed on the ears of laboratory rodents. The main advantage of ear tags is that we can order them in advance with the necessary numbers or letters. On the other hand, the main disadvantage is that the ear tags are so small (approximately 5 mm long) and the experimenter has to immobilize the mouse or rat to read the ID number on the ear tag. You also have to pay attention to the appropriate placement of ear tags. If you put it too close to the edge of the ear, the animal will easily tear it out from the ear (Fig. 17).

Fig. 17 Ear tagging method for marking and identification of laboratory rodents. Created with BioRender.com.

Indirect marking of animals

There is another commonly used way of indirect marking of animals with cage cards. In an animal house, not just the animals have to be marked, but also, their home cages. For that reason, our laboratory uses cage cards with relevant information regarding the animals in particular projects such as:

- PI name,
- Project title,
- Rack+ position,
- Strain,
- Sex,
- Date of birth (Age),
- ID of animals,
- Number of animals in cage (Tab. 10).

Tab. 10 Cage card used at the animal facility at Faculty of Medicine, Comenius University, Bratislava.

Rack + Position:	**Projekt/Responsible person:**
Strain:	**Notes:**
Number + Sex:	
Date of birth:	
ID:	

7.4 Restraint of laboratory rodents (Veronika Borbélyová)

Restraint is defined as an immobilization of a conscious animal by keeping the animal in a fixed position for some time while some examination or procedure is carried out. Restraint minimizes the movements of a laboratory animal but still allows it to breathe normally. You can immobilize the animal either **by your hands** or using some **devices**, e.g., immobilization tube (when measuring the blood pressure of rats and mice, or taking blood from the tail vein of animals). Gentle release of the animal following the immobilization into the experimenter´s hands before the return of the animal to its home cage could help with the adaptation of the animals to restraint. Any form of restraint can be stressful for the animal, thus, its frequency and duration should be kept to an absolute minimum.

7.4.1 Restraint of laboratory mice

Brief protocol for the restraining of laboratory mice **by hand**:

1. Grip the mouse at the base of its tail and gently put the mouse from its home cage out onto the grid of the cage top.

2. Gently grasp the tail of the mouse backward, which leads to the animal moving forward and tending to hold at the cage grid with its forelegs.
3. At this moment, gently press the back with your other hand and approach the rear of the neck and grasp the skin with your thumb and forefinger, while the rest of the skin is held with your other fingers.
4. Pay attention to gripping the skin in the rear of the neck properly, to prevent the turning of an animal's head and biting into your fingers.
5. Grip the tail of the mouse between the third finger and your thumb.
6. Turn your hand upwards to see the ventral side of the body of the mouse.

In this position, the mouse is held safely for other manipulations – sex determination, intraperitoneal injections, or oral gavage of solutions.

Brief protocol for the restraining of laboratory mice **using an immobilization tube (so-called restrainer)**:

Mouse restraining devices can be plastic, leather, hard plexiglass, and metal among others. The restraining device helps the experimenter to have both hands free for the manipulation with the animal. At IMBM, we use mainly plexiglass and plastic type of immobilization tubes.

1. Take the mouse out of its home cage as described above and place it on the table.
2. Hold the mouse gently at the base of the tail and guide the mouse to the open end of the immobilization tube. (If the other end of the immobilization tube is also released, the mouse will enter the tube faster because of its curiosity).
3. Close one end of the immobilization tube, where the mouse has its head, and also the other end, through which the tail of the mouse is exposed.
4. Pay attention to closing the tail side of the immobilization tube in such a way that does not allow movement of the mouse inside of the immobilization tube.

This method of restraint of the mouse allows us to e.g. intravenous administration of substances, measure blood pressure or take blood from the tail vein of the mouse.

7.4.2 Restraint of laboratory rats

Methods of restraint or immobilization for laboratory rats are similar to those for laboratory mice. However, the way of immobilization by hand described above in mice, in the case of rats, is possible only in smaller rats (maximally up to 300 g). Because of the very similar protocols using your hands

and also immobilization tubes (in the case of laboratory rats the tubes are larger) we are going to focus on another way of immobilization of laboratory rats.

Young, but also, adult rats can be lifted from the cage by grasping a rat around its body using one hand over the back of the laboratory rat and placing your thumb under the animal between its front legs (Fig. 18). Exactly your thumb controls the animal's head and restricts its movement to prevent any biting. However, using this method of immobilization also requires some skill as you have to pay attention while you control the movement of the head of the animal, that you leave the animal's ability to breathe. If you lifted the rat from its home cage it is better to provide your other hand underneath the rat's body and move with the rat to the desk, or another cage.

Fig. 18 Grasping a laboratory rat around its body to remove it from the cage. Photo by Veronika Borbélyová.

7.5 Routes of administration of substances (Veronika Borbélyová)

Administration of substances is one of the basic methods in animal experiments. The experimenter is responsible for ensuring that laboratory rats or mice feel as little discomfort or pain during the administration of substances as possible. Thus, the time of administration of substances should be kept at a minimum. There are many routes of administration of substances to laboratory animals, but before administering any substance it is very important to consider its properties such as absorption, bioavailability, metabolism, and excretion. Other factors that must be considered are as follows: toxicity, solubility, frequency and duration of administration, the volume of administration, pH (e.g., rats tolerate a pH between 4.5-8), stability, vehicle, and also, injection techniques (size of needles and syringes). Using the appropriate size of needles and syringes during substance administration could reduce the stress and pain of laboratory rodents.

The routes of administration of substances can be divided into two main groups:

Enteral administration: it means the administration of substances through the gastrointestinal tract (e.g., mixing the substance with food or water of laboratory animals; oral, sublingual administration, or by gavage using stainless steel feeding needles).

Parenteral administration: it means the administration of substances other than via the alimentary canal to the body including injections, infusion, inhalation, implantation of osmotic pumps, etc. The most common and major routes of parenteral administration are subcutaneous, intraperitoneal, and intravenous injections. Other routes of parenteral administration are intramuscular (more in rats than mice), intradermal, intrathecal (application of substances to the spinal cord), intracerebral (into the brain), and intracerebroventricular (into the cerebral ventricles).

During the administration of substances, the animals need to be immobilized properly, and some routes of administration such as intrathecal, intracerebral, and intracerebroventricular require anesthetized animals. The experimenter is responsible to choose the method of administration of substances that cause the lowest stress and pain for the animal. The next section will be devoted to the most commonly used routes of substance administration in laboratory rodents at IMBM.

7.5.1 Oral administration (per os) of substances

It is the simplest route of substance administration when you add the substance to the food or drinking water of the animals. However, in that case, you have to monitor the daily food and water intake before and during the experiment to calculate the consumed quantity of the substance. The disadvantage of this route of administration is that food and water wastage happen all the time, thus, it is difficult to determine the precise amount of food and water intake, therefore, the precise intake of the substance.

7.5.2 Intragastric administration of substances by oral gavage

Direct administration of substances by oral gavage is the most accurate method of intragastric administration of substances. However, the experimenter needs to be skilled in the proper immobilization of laboratory animals. This method of administering the substance is carried out using the so-called oral probe – a bent stainless feeding needle with a ball end. The advantage of this method of administering substances compared to oral (per os) administration is that we know the exact dose of the administered substance directly into the stomach, which absorption is faster than when the substance is administered in food or drinking water.

Brief protocol of oral gavage:

The first step is to choose a probe of appropriate size for rats or mice. Afterward, take the given amount of the substance to the syringe with a probe.

1. Restrain the conscious mouse or rat properly by gripping a fold of the skin from the scruff of the neck down the back.
2. Immobilize the head of the animal – this is crucial for successful oral gavage.
3. If you extend the neck and the head of the animal properly a straight line is formed between the mouth and the stomach.
4. Gently pass the needle through the mouth and pharynx into the esophagus. The animals usually swallow the feeding needle when it approaches the pharynx, and this could help to get the probe through the esophageal opening.
5. Administer the substance slowly.
6. Remove the probe slowly.

If you feel any obstruction e.g., the animal starts to cough, or choke or you see fluid coming out from the nose, these may indicate that the needle has entered the lungs. In this case, carefully remove the probe and put it in the mouth again, adjusting its position.

7.5.3 Intraperitoneal administration of substances

Intraperitoneal administration of substances is the most common route of administration being technically simple and easy. The advantage of this route of administration is that you can apply a relatively large volume of a dissolved substance, but the rate of absorption by this route is usually one-half to one-fourth as rapid as from the intravenous one.

Brief protocol of an intraperitoneal injection:

1. Restrain the conscious mouse or rat properly, thus, hold the animal in a supine position = the head and the body of the animal are in a horizontal position and the abdominal area is facing us.
2. Push the needle in at approximately 10° angle between the needle and the abdominal surface in the lower quadrant of the abdomen. Intraperitoneal injection is given to animals usually into the right lower quadrant of the abdomen due to the absence of anatomically important structures or organs on this side of the abdomen.
3. Administer the substance slowly.
4. Remove the needle slowly.

If you need to inject substances repeatedly through several days or weeks, alternate the site of intraperitoneal injection daily.

7.5.4 Subcutaneous administration of substances

Subcutaneous injections are rarely painful, which is why a conscious animal can be used. However, the rate of substance absorption is lower than in the case of intraperitoneal and intramuscular injections. Subcutaneous injections are usually made into the loose skin in the interscapular area (Fig.

19 A) or at the inguinal area (Fig. 19 B). This route of administration is preferred when small volumes of substances (aqueous or oily fluids) are given to laboratory rodents.

Fig. 19 Subcutaneous administration in interscapular area (A) and inguinal area (B). Created with BioRender.com.

Brief protocol of a subcutaneous injection into the interscapular area of a rodent back:

1. Restrain the animal and then place the animal on a clean towel or solid surface.
2. Insert the needle under the skin in the interscapular area while holding the skin between the thumb and forefinger.
3. Inject the substance slowly.
4. Remove the needle slowly.

7.5.5 Intravenous administration of substances

The main advantage of intravenous administration of substances is that compounds that are poorly absorbed by the digestive tract may be given intravenously. However, this route of administration requires to have real technical expertise and skill. Usually, the lateral tail vein of laboratory rodents is used for intravenous injection of substances, as the dorsal tail vein is not straight. You can perform intravenous administration either in a conscious animal or an anesthetized one. If anesthesia is not used, a restrainer is necessary for the immobilization of the animal.

1. First, the syringe with the needle has to be filled with the solution and air bubbles have to be removed. This is important to prevent embolism. Air bubbles in the syringe or the fluid can be removed by a gentle tapping of the side of the syringe and slowly expelling the air bubble through the needle.

2. Place the conscious animal into the restrainer.
3. Warm the tail of the animal using a lamp or warm towel, or you can also immerse it in 40 - 45 °C warm water. This step is crucial to dilate the tail vein.
4. Disinfect the tail of the rodent with 70% alcohol on a swab.
5. Insert the needle parallel to the tail vein while keeping the bevel of the needle face upwards (Fig. 20).
6. Slowly inject the solution and no resistance should be felt.
7. Slowly remove the needle, and while removing press firmly the injection site with a swab or fingers.

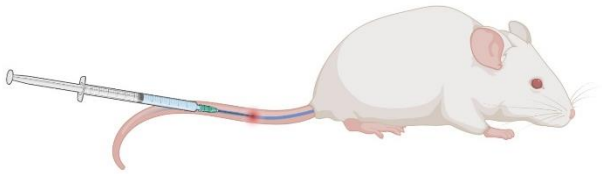


Fig. 20 Intravenous administration of substances. Created with BioRender.com.

Volume and frequency of administration of substances

In general, the rate of absorption of substances is arranged in the following order: **intravenous administration > intraperitoneal administration > intramuscular administration > subcutaneous administration > oral administration.**

The volume of the administered substance is limited by any toxicity of the substance and also by the size (body weight) of laboratory rodents (Tab. 11). It can vary on the species (rat versus mouse), strain, route, and frequency of administration, but also, with the composition of the solution. The volume of substance administration should generally be kept as small as possible, as the application of a large volume may cause pain and stress to the animal. Also, the frequency of administration should be reduced to a minimum to reduce the stress of the animal. Another important thing is the adequate speed of administration of the substance. In general, injecting substances should be a slow process, but we can perform intraperitoneal, intragastric, and subcutaneous administration of substances in a faster way than e.g. intravenous injection.

Tab. 11 Recommended doses (ml/g) and needle sizes (G) according to the route of administration of substances to mice and rats.

	Laboratory mouse		Laboratory rat	
Route of administration	**Recommended dose**	**Needle size**	**Recommended dose**	**Needle size**
Intramuscular	0.00005 ml / g	< 23 G	0.1 ml / g	< 21 G
Intraperitoneal	0.02 ml / g	< 21 G	10 ml / g	< 21 G
Subcutaneous	0.01 ml / g	< 22 G	5 ml / g	< 22 G
Intravenous	0.005-0.025 ml / g	< 25 G	5-20 ml / kg	< 23 G
Oral gavage	0.01 ml / g	20-22 G	5-10 ml / kg	16-20 G

7.6 Blood collection from laboratory rodents (Ľubica Janovičová)

Blood collection in laboratory rodents is part of all experiments carried out in our laboratory. Blood can be collected during the experiment duration and at the end when rodents are sacrificed. Blood collection should be carried out in such a way that it minimizes the stress in rodents and blood volume suffices for the required experiments. However, several aspects need to be addressed before collecting blood. Among these are:

1. **Collected blood volume:**
   When planning an experiment, it is crucial to consider selected animal models based on markers that need to be measured, and the ability to do surgeries or treatment in either rats or mice. The blood volume of mice and rats can be estimated based on their body weight. Rats have approximately 64 ml of blood per kg of body weight. That means that the estimated total blood volume of 200 g rat is 12.8 ml. In mice, the estimated blood volume is 77 to 80 ml per kg of body weight. This means that a mouse with a body weight of 20 g had approximately 1.5 to 1.6 ml of blood. However, it is not possible to collect the total volume of blood from laboratory rodents. For blood sampling that is not terminal, it is recommended to collect a maximum of 10% of the total blood volume of both mice and rats. That would represent approximately 1.2 ml of blood from 200 g rat and 150 ul of blood from 20 g mouse. Blood

volume is recovered in 24 hours but the lost cells in the blood such as erythrocytes may take longer to return to normal counts. Therefore, it is recommended to collect the blood for repeated blood sampling once every two weeks. If a larger volume of blood is collected due to some specific animal model requirements it is suggested to closely monitor the well-being of mice throughout the experiment.

2. **Planned measurements from blood:**

It is crucial to plan all measurements that should be output from the blood that is collected. This is especially important if repeated blood sampling is done in mice, where small volumes of blood are obtained. No more blood should be collected than is permitted to avoid inducing stress or anemia in laboratory rodents.

Before collecting blood, the need for anticoagulants should be determined based on the intended measurement. The frequently used anticoagulants are:

- EDTA
- heparin
- citrate

Tubes for blood collection differ in the size too. This means that depending on blood volume tube size is selected as it already contains a certain amount of anticoagulant (Fig. 21). In some cases, a serum is collected in tubes that do not contain any anticoagulant. Blood is left to coagulate and then centrifuged.

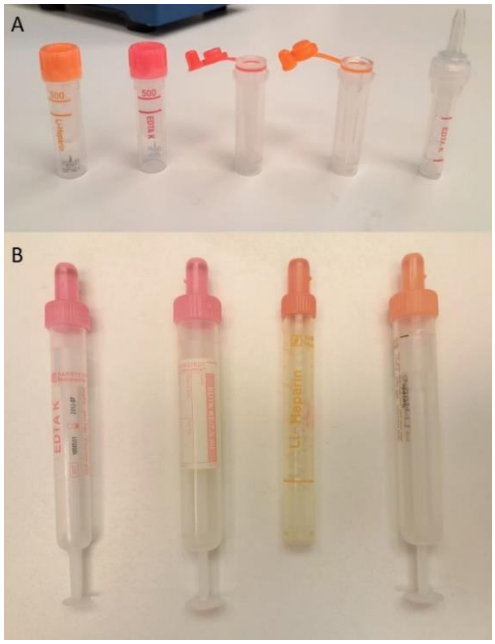


Fig. 21 The most commonly used tubes for blood collection from mice and rats in our laboratory. A) smaller microvette tubes for up to 0.5 ml. B) larger monovette tubes for up to 6 ml of blood. Photo by Ľubica Janovičová.

Blood collection methods used in our laboratory:

1. **Anesthesia not required:**

   Tail snip

   This method is commonly used in both laboratory mice and rats. This way a mixture of arterial and venous blood is obtained. For repeated blood sampling in a short period of time, it is sufficient to remove the clot only with a tampon or tissue. The tail tip does not need to be cut off again. This blood collection method is ideal if there is no need for larger blood volume collection and only smaller volumes are collected.

   Procedure:

1. To immobilize the animal, place it in the immobilization tube (Fig. 22).
2. Using sharp scissors, cut off a small piece from the tip of the tail (0.5 - 1mm).
3. Collect drops of blood in microvette tubes. The cap and bottom closure of the tube need to be open. Make sure not to tilt the tube too much, otherwise, the blood may spill from the tube.
4. Cap the bottom and top of the tube and place the collection tube in the clear cylinder-shaped part before sample processing.

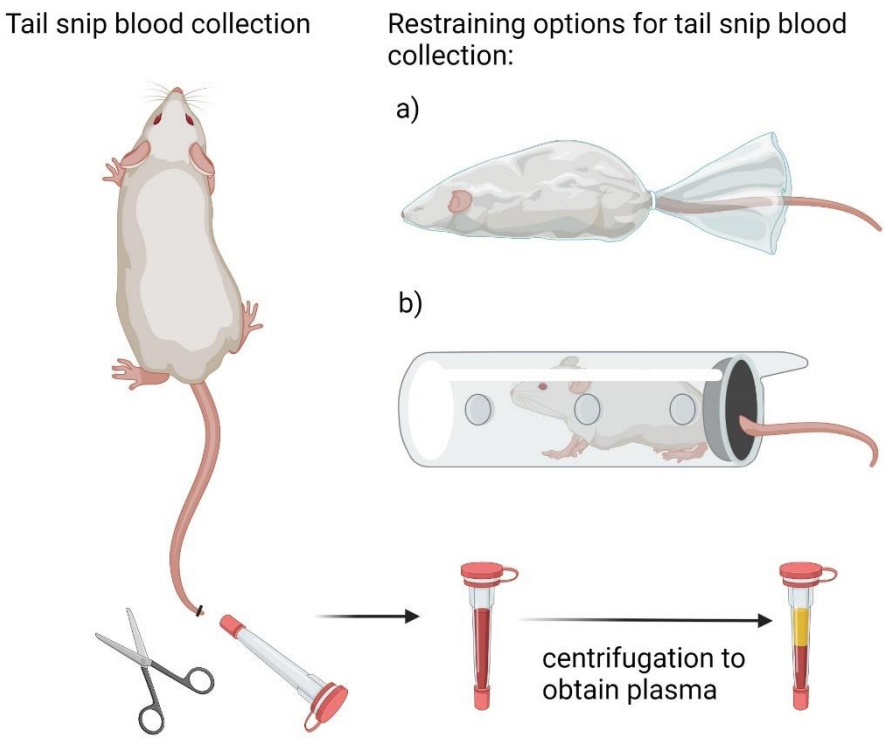


Fig. 22 Tail snip blood collection. Created with BioRender.com.

2. **Anesthesia required:**

Retro-orbital plexus puncture

This technique is one of the most commonly used methods of blood collection in laboratory rodents. Animals must be under general anesthesia. Inhalation anesthesia with isoflurane is used to briefly anesthetize animals for blood collection.

Procedure:

1. Place the animal in a prone lying position. The mouse is held so that the head is angled in such a way the eye is accessible and the eye slightly bulging.

2. The glass capillary is placed in the front of the inner corner of the eye at an angle of 30 - 45°.

3. Gently turn the glass microcapillary with a slight pressure between the thumb and forefinger until the orbital sinus is reached.

4. As soon as the right place is reached, blood flows through the glass capillary. Blood is collected into the microvette tube by letting it drip from the end of the glass microcapillary.

5. After blood collection, we remove the glass microcapillary from the eye, close its eyelids, and using a piece of gauze gently press on the eye to stop the bleeding (Fig. 23).

Cardiac puncture

In this method of terminal blood collection, the animal must be under terminal anesthesia. There are several options on how to access the heart to do the cardiac puncture. It can be done with and without thoracotomy.

Procedure:

1. Anesthetized mouse is placed in a supine lying position.

2. Using a 1 ml syringe with a 22G needle a puncture is made 5 mm from the center of the chest towards the chin of the animal. The syringe is angled 25-30° from the chest. If the cardiac puncture is done with thoracotomy, the heart ventricle is punctured with a needle, and blood is collected.

3. When blood appears in the syringe, the plunger should be gently pulled to collect as much blood as possible and prevent hemolysis. Do not pull the syringe plunger with force, that may result in heart collapse.

4. If the blood stops flowing, repositioning the needle can help collect more blood.

5. After collecting blood, the needle is removed from the syringe. Blood from the syringe is then transferred into a tube. If blood with anticoagulant should be collected, the anticoagulant should be drawn into the syringe before the blood collection (Fig. 23).

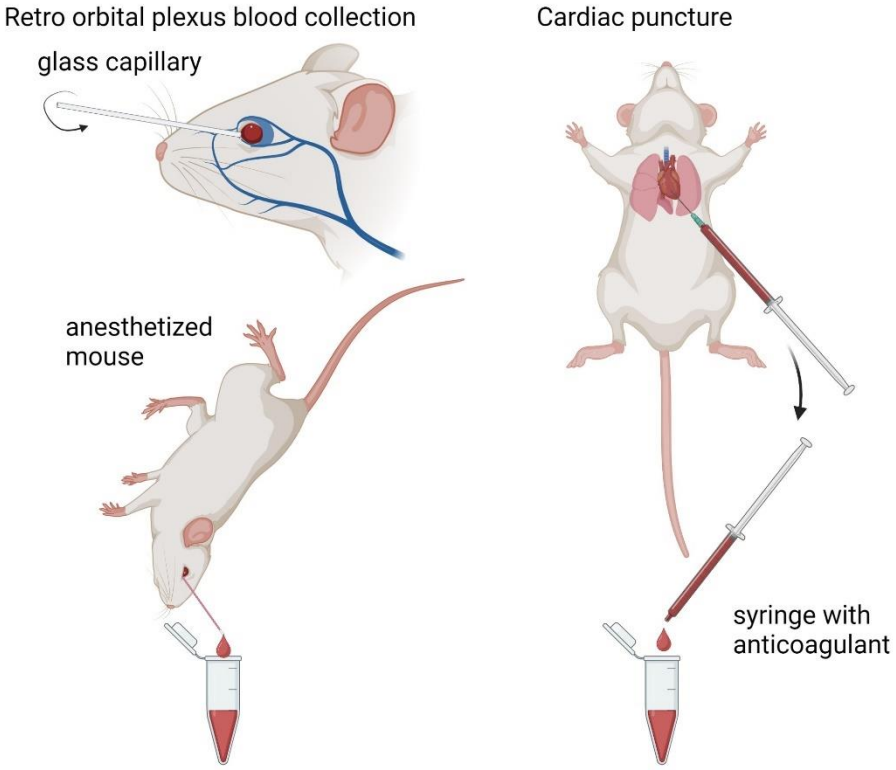


Fig. 23 Blood collection from retro-orbital plexus and cardiac puncture. Created with BioRender.com.

8 Behavioral characterization of laboratory rodents (Alexandra Gaál Kovalčíková)

Ethology from the Greek words ethos = behavior, logos = study is a scientific discipline dealing with the behavior of animals and humans, especially in their natural environment. If you are investigating the behavior of animals, you have to know the natural behavior of the animal to be able to recognize changes or alterations in animal behavior. Animal experiments allow us to investigate the behavior of animals in detail, however, in that case we analyze the behavior of animals in laboratory conditions.

Ethology is divided into:

● **descriptive ethology** – description of the elements of animal behavior based on direct or indirect observation.

● **experimental ethology** – formation of clear hypotheses that are the basis for further analyses.

In general, the researcher does not monitor the whole behavioral repertoire of an animal, but he focuses on the observation of a given form of behavior of laboratory rodents according to the goals of his project. Currently, the basic behavioral repertoire of laboratory rats and mice is well known. Laboratory rats and mice are nocturnal and social animals, for that you can pay attention. If you want to observe the behavior of rodents during their active (dark) phase, you have to not observe their behavior in their passive (light) phase of the day. As laboratory rats and mice are social animals, you have to ensure the animals have cage mates and do not separate them alone to cages if it is not necessary (e.g., collection of fecal samples, individual measurement of food intake, etc.), as it can affect the results of your experiment.

Laboratory rats and mice show a physiological similarity to humans, which is why they are commonly used animal species in the scientific field. If you investigate the behavior of laboratory animals as part of your experiment, you should always have a so-called control group. According to the behavior of the control group of rats or mice, you can define changes in the behavior of your experimental groups of animals.

In the home cage environment, we can observe many behavioral parameters (e.g., self-grooming, climbing on the cage grid, rearing, social contact, etc.) of laboratory rodents, which do not require any special equipment. However, observation of the complex behavior of animals needs using of the so-called mazes, which allows testing a complex behavioral repertoire of laboratory rodents, however, outside of their home cage.

The next chapters are devoted to behavioral tests (mazes) used for assessing locomotor activity, anxiety- and depression-like behavior, social behavior, communication, repetitive behavior, spatial orientation, and memory of laboratory rodents. These tests are commonly used at IMBM. Behavioral tests are conducted in a separate room (Mazes room). An important part of behavioral testing for the reduction of stress from a new environment is habituation. It is necessary to habituate the animals in the Maze room for 30-60 minutes before the test (if the specific test does not require otherwise). A lot of the behavioral tests (e.g., the open field test, 24 hours monitoring, Reciprocal social interaction test, and Novel object recognition test are conducted in special cages called PhenoTypers (Fig. 24). It is a square cage with transparent walls. The camera is above the cage, and it records the animal's behavior for a given time period. The PhenoTyper cage is connected to the computer. For recording and data analysis the EthoVision XT 16 (Nodus) is used. Is important to keep in mind that the floor of PhenoTyper is changeable and it has to be in contrast with the color of the testing rodent. The cleaning and changing of the bedding are important after every single tested animal.

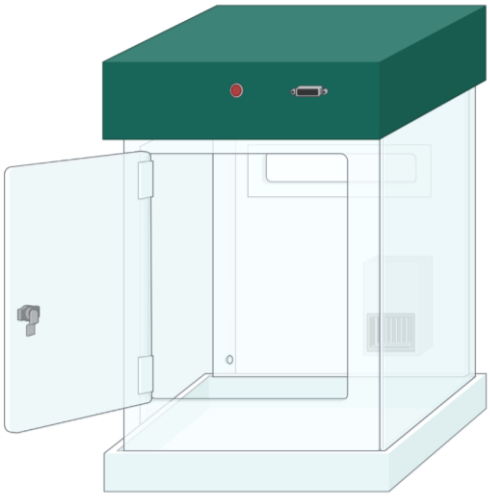


Fig. 24 Scheme of the Phenotyper cage. Created with BioRender.com.

8.1 Locomotor activity of laboratory rodents (Petronela Sušienková)

Open field test

The open field test (Fig. 25) measures locomotor- and exploratory activity, anxiety-like behavior, grooming, and rearing of laboratory rodents. The rodent is inserted in the central zone of the PhenoTyper cage (for rats: 100 cm × 100 cm; for mice: 45 cm × 45 cm). The movement of the animal is recorded for 10 minutes. The following parameters are analyzed: distance moved and average

velocity of the movement (locomotor activity), time spent in the central zone (anxiety-like behavior), grooming (repetitive behavior), and rearing (exploration).

Fig. 25 Scheme of the open field test. Created with BioRender.com.

8.2 Long-term observation of the rodent behavior in PhenoTyper cages

To observe the behavior of rodents in a specific phase of the day, 24-hour homecage monitoring of the animal behavior should be used. The monitoring of behavior, in that case, is conducted in PhenoTyper cages with bedding, sleeping area (plastic house), food, and water (Fig. 26). The locomotor activity, sleeping behavior, eating, drinking, rearing, grooming, time and frequency of entries into the central and border zone of the area are observed.

Fig. 26 Long-term homecage monitoring of the behavior of mice in PhenoTyper cage equipped with bedding, mouse igloo-house, food, and water bottle. Created with BioRender.com.

The results from the test may be visualized as an ethogram. The Fig. 27 shows the report of the observed behavioral parameter during long-term monitoring of rodent behavior. Another option is a heat map (color visualization of the time spent in specific areas of the PhenoTyper cage) as you can see in Fig. 28.

Fig. 27 Ethogram of 24-hour homecage monitoring of mouse behavior.

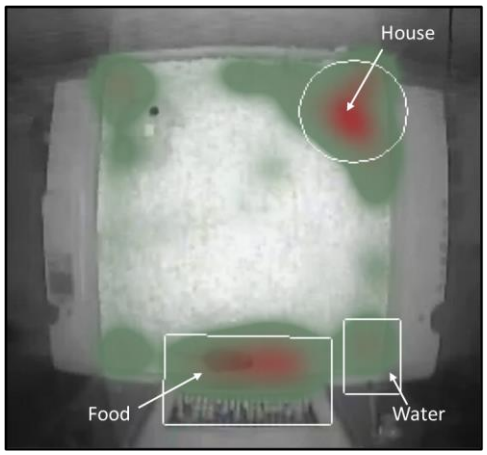


Fig. 28 Heatmap of 24-hour home-cage monitoring of mouse behavior.

8.3 Anxiety-like behavior of laboratory rodents

Elevated plus maze

The elevated plus maze is used for the evaluation of the anxiety-like behavior of laboratory rodents. The maze consists of two open and two closed arms (Fig. 29). The open arms are light, and the closed arms are covered by walls. The maze is elevated 60 cm above the floor. The rodent is inserted in the central zone of the maze facing an open arm and is allowed to freely explore the maze for 5 minutes. Many variables may be observed in this test: time in the central zone, time in open arms, time in closed arms, number of entries into the open arms, and the number of entries into the closed arms. An anxious animal will spend less time in the open arms and enter the open arms fewer times than the closed arms.

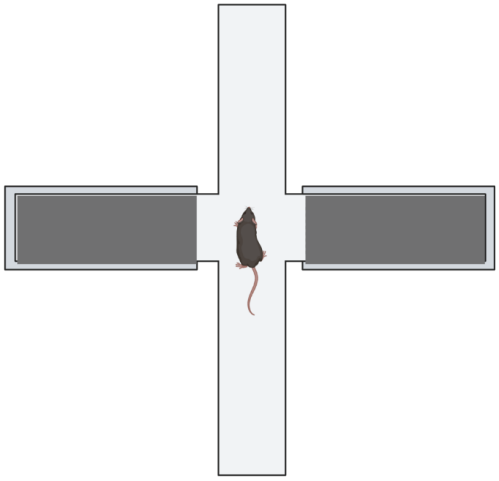


Fig. 29 Scheme of the elevated plus maze test. Created with BioRender.com.

Light-dark box

For testing anxiety-like behavior the light-dark box should be also used. The maze has two same-size chambers (Fig. 30). One of them is light and the other is dark. The animal is inserted into the light chamber, and it can walk freely through the chambers for 5 minutes. More time spent in the light chamber is associated with lower anxiety-like behavior.

Fig. 30 Scheme of the light-dark box. Created with BioRender.com.

Open field test

Besides the locomotor activity measurement, the open field test could be used also for assessing the anxiety-like behavior of laboratory rodents. The more time spent in the center zone and the higher number of entries to the center zone reflect lower anxiety-like behavior.

8.4 Social behavior of laboratory rodents

Reciprocal social interaction test

Laboratory rodents are social animals. This test is used for the evaluation of the social behavior and reactions within social interactions of laboratory rodents. If you are going to perform this test, to increase the need for socialization in animals, you have to socially isolate them for 24 hours before the test in separate cages. The testing animal and unknown social partner are located in the PhenoTyper cage with bedding (Fig. 31) for 10 minutes. The characteristics of social partners (such as age, sex, and genotype) are different according to the aims of the given projects. The records of the test are scored manually, and you evaluate the parameters as follows: social interest (nose-to-nose sniffing, nose-anogenital sniffing, side sniffing, following, keeping pace, non-aggressive mounting, social grooming, playing), social disinterest (self-grooming, digging, lying flat, freezing in contact, avoiding the contact, non-social rearing, evasive behavior), and aggression (biting, wrestling, pushing, aggressive mounting and rearing). As the evaluation of these parameters, you do manually from video recordings, it is necessary to score the test by at least two independent observers. Final data for analysis are calculated by the mean of the time measured by observers.

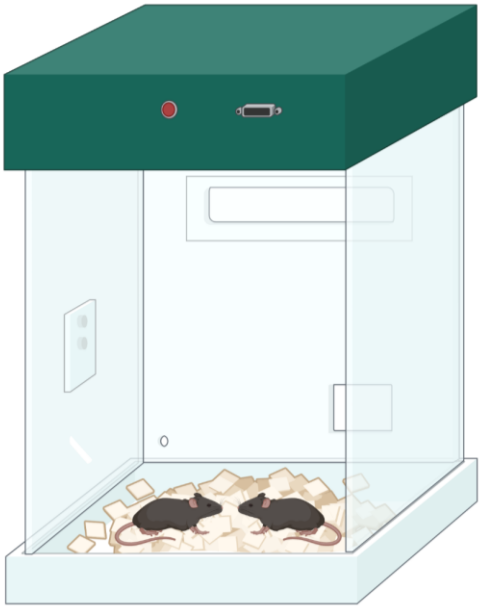


Fig. 31 Scheme of reciprocal social interaction test in PhenoTyper cage. Created with BioRender.com.

Three-chamber sociability and social novelty test

For analysis of social preference and social novelty preference, you can use the so-called three-chamber social interaction test. The apparatus consists of e.g. for mice a polycarbonate box (60 cm × 40 cm) with three chambers of equal size (20 cm × 40 cm). The middle chamber is equipped with two removable openings that allow free access of animals to side chambers. During a habituation session (10 min), the subject mouse is placed in the middle chamber with access to each side chamber containing empty plastic containment cups. In a social affiliation session (10 min), an unfamiliar mouse (stranger) is placed inside one plastic containment cup, while the same but empty plastic containment cup is positioned in the opposite chamber (Fig 32a). Unfamiliar social partners are in general from the same strain, age, and sex as the tested mouse. Sociability is quantified as the time that the tested mouse spent sniffing the cup with a stranger versus the empty containment cup. The time difference between them points out social preference. Another option is a calculation of the social preference index using this formula:

$$Index\ of\ social\ preference = \frac{(time\ of\ sniffing\ social\ partner\ -\ time\ of\ sniffing\ object)}{total\ time\ of\ sniffing}$$

For testing of social novelty preference, the non-social object is replaced by another unknown animal. The social partner from the first part of the testing trial stays in the cylinder and it should be recognized by the testing animals from that trail (Fig 32b). Now, there are two social partners in the cylinders: one is already a familiar one in one chamber, and in another chamber, there is a new unknown social partner. The difference between the time spent sniffing unfamiliar and familiar social partners is measured. If the animal spends more time sniffing the unknown social partner, it is an indicator of social novelty preference.

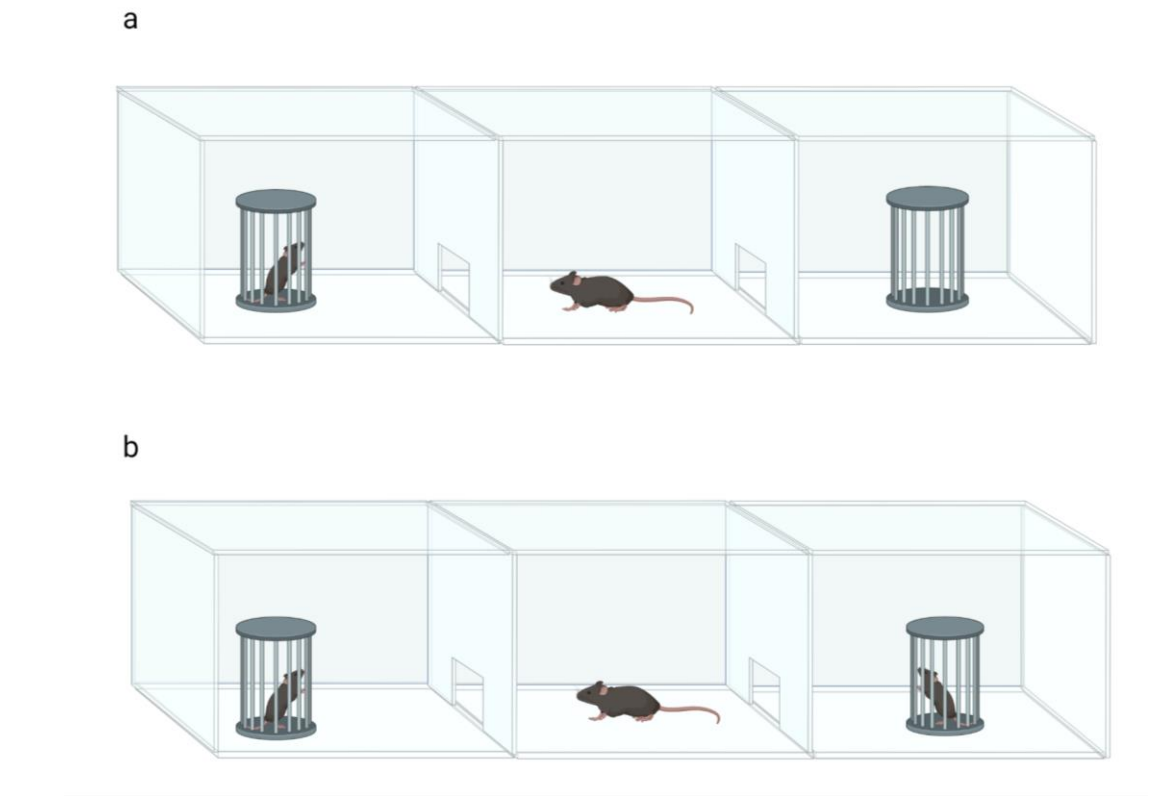


Fig. 32 Scheme of the three-chamber sociability and social novelty test. Created with BioRender.com.

Some important points need to be followed. Habituation of all animals, including social partners, is necessary before testing them in this test. Keep in mind that the social partners have their physiological needs such as drinking and eating. For that reason, the social partners should be changed during the testing period. Thirdly, the social partner should be the same strain and age. Depending on the aim of the experiment the social partner may be male or female.

8.5 Depression-like behavior of laboratory rodents

Forced swim test

During the forced swim test, animals are individually placed into a plastic cylinder (height 45 cm, diameter 30 cm) filled with tap water (24-25 °C, Fig. 33). Time spent with immobility is recorded during the 6 min testing period (in mice) and 5-15 min testing period (in rats) as an index of depression-like behavior. The first two minutes of the testing period in mice is considered a habituation period and only the last four minutes of the test are analyzed. After the test, the animal is drained, and the cage with animal is located under infrared light.

Fig. 33 Scheme of the Forced swim test. Created with BioRender.com.

8.6 Spatial learning and memory of laboratory rodents

Novel object recognition test

For testing the memory of laboratory rodents, the novel object recognition test can be used. This test consists of a familiarization session with the maze used for the Open field test (PhenoTyper cage), the first phase of testing, retention, and the second phase of testing. Firstly, the animal is in an empty PhenoTyper cage to explore it for 5 minutes – familiarization session (Fig. 34a). The testing session starts with the first trial (5 min) when the animal is placed into the cage where two new objects are presented in opposite corners (Fig. 34b). After that, the animal is moved to the home cage for one hour (retention). Finally, after the retention period, the animal is removed from the home cage to the testing cage where there is already one known object and one new object (Fig. 34c). The exploring of objects (number of sniffs and time of sniffing) is measured in this test. If the rodent remembers the known object in the second phase of testing, it should spend more time exploring the new object.

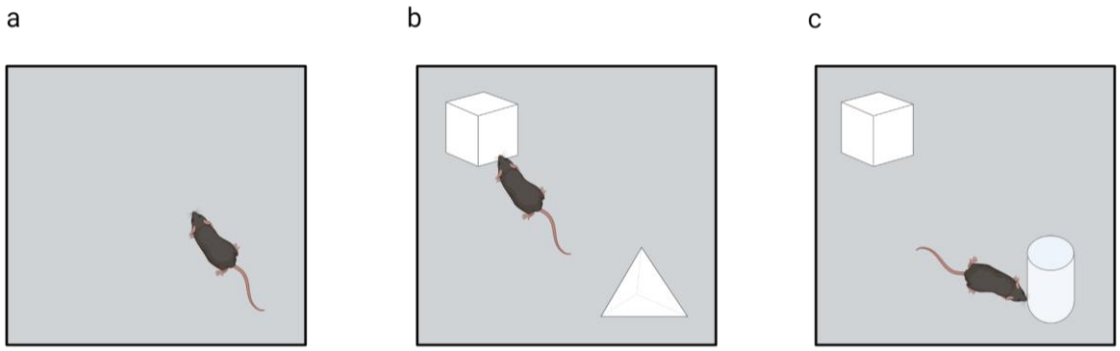


Fig. 34 Scheme of the novel object recognition test (a. habituation; b. unknown objects; c. one known and one novel object). Created with BioRender.com.

Morris water maze

For measuring spatial memory and learning the Morris water maze test is used. The dark circle pool is virtually divided into 4 same-size quadrants marked by different symbols (Fig. 35a). The pool is filled with 24-26 °C water. In one of the quadrants, there is a platform 2 cm under the water. The test aims to evaluate the ability of laboratory rodents to remember the location of the platform based on the orientation according to symbols on the wall of the pool (Fig. 35b). The mouse or rat should find the platform in maximally 60 seconds. If the mouse or rat is not successful in 60 seconds, it is gently moved to the platform by the experimenter and the animal stays on the platform for 30 seconds to remember it (working memory). The animals are tested four times in one day, and they start their swim in each quadrant facing the symbol. During 4 testing days, the animals should learn the location of the platform. The variables in this phase of the Morris water maze test escape latency = time that the animal needs to find the platform in each trial and the swimming distance. After 4 day learning phase, the platform is removed from the pool, and the probe trial is conducted. The time spent in the quadrant where the platform was in the learning phase is measured as a parameter of long-term memory in the probe trial.

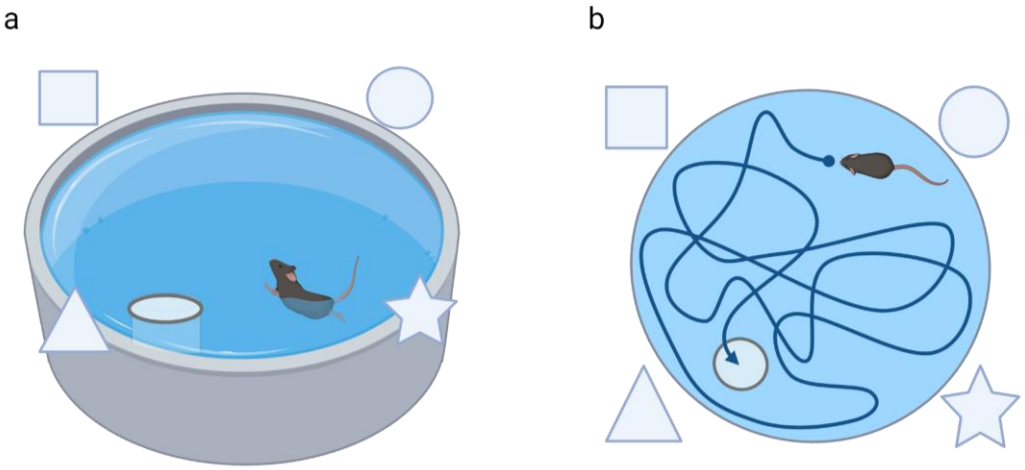


Fig. 35 Scheme of the Morris water maze test. Created with BioRender.com.

8.7 Repetitive behavior of laboratory rodents

Marble burying test

The test measures repetitive behavior and object avoidance in rodents. 5 cm of the bedding is placed into the cage before the test (Fig. 36). The glass marbles (numbers: 12 - 25) are laid out in rows and columns symmetrically. The animal is inserted in the cage for 20 minutes. After that, the number of buried marbles (75 % of the marble area is buried) is counted manually which is a variable of repetitive behavior. A low number of buried marbles suggests object avoidance.

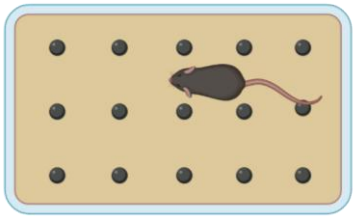


Fig. 36 Scheme of the marble burying test. Created with BioRender.com.

Open field test

To monitor the repetitive behavior of mice, self-grooming behavior is manually scored by an experimenter during the testing in the open field test. The number and duration of grooming events in each mouse/rat are quantified during the 5-10 min testing period. The presence of increased self-grooming indicates repetitive behavior.

8.8 Social communication in laboratory rodents

Laboratory mice and rats use ultrasonic vocalizations (USVs) to communicate with each other and to convey their emotional state. At IMBM, we measure mother isolation-induced USVs for pups or female-induced USVs for male mice during courtship.

Social communication, defined as the number of calls, is the output parameter of the measurement of ultrasonic vocalization. The quantity and quality of the calls may be measured. The testing is conducted in a soundproof plastic box for 5 minutes. The microphone is placed on the top of the box. For recording, UltraVox 3.2 software is used.

In the case of measurement of so-called pup-isolation calls, when the pups are separated from their mother, the number of calls is measured in infant mice or rats (Fig. 37a). During adolescence and adulthood, the rodent mostly vocalizes during social contact, aggressive interactions, or mating (Fig. 37b). In our conditions, we use improved social reciprocity tests with the scoring of ultrasonic vocalization. Animals are separately placed into cages for a minimum of 12 hours of social isolation. After social isolation, animals are placed in a testing room for 30 minutes for habituation to the experimental environment. Then, animals are placed into the arena without sawdust (to avoid noise), the number of calls is measured and videos for social interest and disinterest are manually scored. You have to pay attention to silence in the testing room, because of the high sensitivity of the microphone.

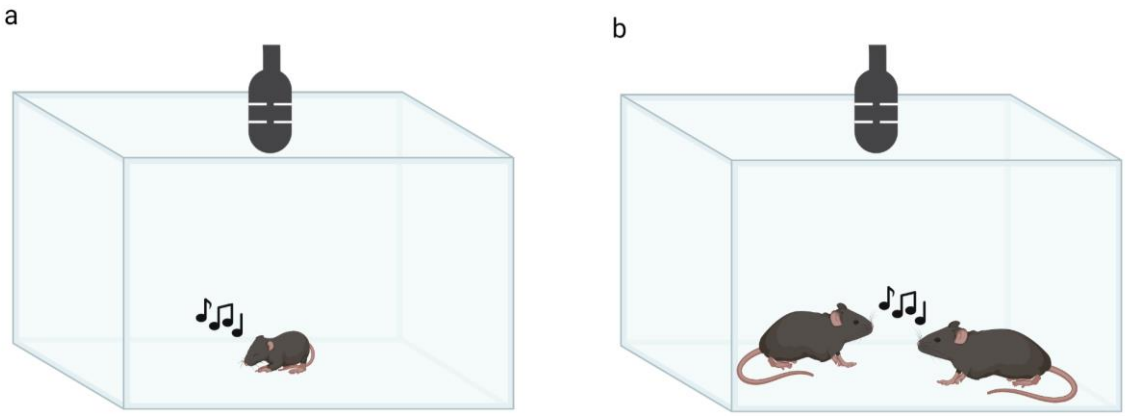


Fig. 37 Scheme of recording ultrasonic vocalization in a pup separated from its mother (a) and during adult social interactions (b). Created with BioRender.com.

9 Measurement of metabolic parameters in laboratory rodents

9.1 Obesity and metabolic syndrome

Obesity is characterized by abnormal accumulation of fat in adipose tissue predominantly in the abdominal region. This pathological body weight gain is associated with the development of metabolic syndrome, which further increases the risk of cardiovascular morbidity and mortality, thus, representing a global health problem. The main components of metabolic syndrome include obesity, high blood pressure, high blood triglycerides, low levels of HDL cholesterol and insulin resistance. For the evaluation of obesity in patients, the calculation of body mass index (BMI) is used. BMI is the weight of the person in kilograms divided by the square of height in meters.

$$\text{BMI} = \frac{\text{body weight (kg)}}{\text{body height (m}^2\text{)}}$$

At our institute we deal with the topic of metabolic syndrome. Please, allow us to explain how to evaluate the components of metabolic syndrome in laboratory animals.

9.1.1 Measuring body weight of laboratory rodents

The easiest option for the evaluation of obesity in laboratory rodents is the observation and measurement of the body weight of the animals. Generally, body weight is measured weekly, at the same time of day period. The reason for performing body weight measurements on the same day time is that rodents had defecation and urination habits and if it is not performed in that way, we can have lower values than it is in reality. When body weight is measured, the person who is performing it needs to think about using the same weight (to avoid technical variability of measurement). The weight needs to be stored on a flat surface. Calibration of the weight needs to be done before measurements. Also, at the beginning, we need to check if the weight is in balance (Fig. 38 C).

Between measurements, we need to press “TARE” on the weight to have zero for the next upcoming measurement.

There is a difference between assessing the body weight of laboratory mice and rats. Generally, weight in scale from 0 to 250 g is used for mice, with the two decimal numbers (Fig. 38 A). Mice are smaller, so, the body weight of mice is assessed for two decimal numbers, as well as their body weight gain is not so obvious during modeling the obesity. The weight in the scale from 0 to 1000 g is used for rats (Fig. 38 B). Generally, we do not measure the body weight of rats for decimal numbers, because of their greater physiological body weight.

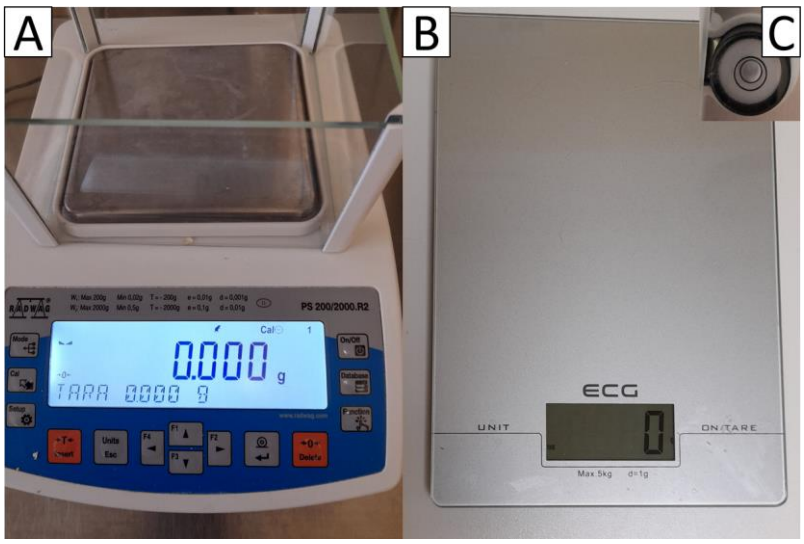


Fig. 38 Weights used in labs of IMBM for the measurement of rodent body weight. A – Analytical weight for mice; B – Precision weight for rats; C – Balancing bubble of analytical weight – has to be centralized at the level indicator. Photo by Andrej Feješ.

9.1.2 Morphometric measurements in laboratory rodents associated with obesity

Obesity as previously described is characterized by the accumulation of fat mostly at the level of the abdomen. For assessing the morphological changes in laboratory rodents, morphometric measurements are used. In general, the waist circumference, naso-anal distance, and total body length are measured (Fig. 39).

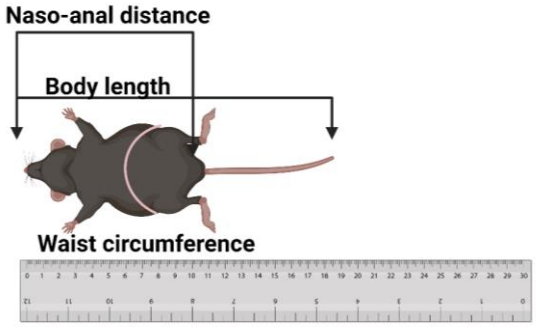


Fig. 39 Morphometric measurements. Created with BioRender.com.

- **Waist circumference** (cm) is measured at the level of the belly button using thread, ruler, and scissors. Approximately 10 cm (mice) or 30 cm (rats) of thread is cut, placed to the last rib in the abdomen region and waist circumference is measured. With the ruler, we gauged the accurate waist circumference.
- **Naso-anal distance** (nose-to-anus body length, cm) is measured as the distance from the nose to the anus. For mice, we use a caliper and we evaluate the distance on two decimals in centimeters. For rats, we are using a ruler in whole numbers and also in centimeters.
- **The total body length** is measured from the nose tip to the end of the tail tip. We use a ruler stuck to the table. Body length is measured in the supine position of the rat/mouse on the laboratory desk.

Obesity evaluation from morphometric measurements can be assessed from waist circumference (central obesity) but also using calculations of obesity indexes. More precise for central obesity evaluation is a calculation of **waist to body length ratio**. This index is calculated as waist-circumference divided by naso-anal distance. Similar to BMI is the **Lee index** used in animals for the evaluation of obesity. The Lee index is calculated as body weight divided by naso-anal distance. In addition, as in humans, the **BMI** might be calculated for laboratory rodents, as the weight of the animal in grams divided by the square of total body length in centimeters. However, BMI is not a perfect index for obesity assessment, because the calculation also contains the tail of the animal, where fat is not present as much.

$$\boldsymbol{Waist/length\ ratio} = \frac{\boldsymbol{Waist\ circumference\ (cm)}}{\boldsymbol{Naso-anal\ distance\ (cm)}}$$

$$\boldsymbol{Lee\ index} = \frac{\boldsymbol{Body\ weight\ (g)}}{\boldsymbol{Naso-anal\ distance\ (cm)}}$$

$$\boldsymbol{Body\ mass\ index\ (BMI)} = \frac{\boldsymbol{Body\ weight\ (g)}}{\boldsymbol{Total\ body\ length\ (cm)^2}}$$

9.2 Insulin resistance in laboratory rodents

Insulin resistance is one of the main components of metabolic syndrome. Insulin resistance means that the body does not use insulin efficiently to lower blood glucose concentrations. In turn, the pancreas will begin to produce more insulin to reduce the blood glucose concentration which leads to hyperinsulinemia. Insulin resistance can be evaluated with an Oral glucose tolerance test. Animals are put on fasting for 12 hours (overnight). The fasting needs to be done in a home cage environment if you do not need to collect urine samples from the animals. In that case, fasting can be also performed in metabolic cages, where urine can be collected during the fasting period, however, it is more stressful than fasting in a home cage environment. Before the placement of animals for fasting, new cages with clean sawdust need to be prepared, since coprophagy during starvation is typical in laboratory animals. Check animals to have free access to water.

Before the oral glucose tolerance test, the glucose solution in a concentration of 2 g / kg of body weight needs to be prepared for oral gavage. You have to weigh the glucose on analytical scale and dissolve it in tap water.

Brief protocol for glucose solution preparation:

1. Animals are weighed after fasting since the dose of glucose is administered based on body weight.
2. Prepare stock glucose solution.
3. If we know that the dose is 2 g / kg, we need to weigh 20 g of glucose and dissolve it in 20 ml of tap water.
4. The final concentration of glucose in the stock solution will be 1 g/ml, which represents a 2 g / kg dose.
5. Then we will calculate the volume of glucose for every single animal.

For example:

Dose……2 g glucose……..1000 g……1 ml

Animal weight……2 g glucose……..……400 g……x ml

$$400 : 1000 = x : 1$$

$$400 = 1000x$$

$$\mathbf{x = 0.4\ ml}$$

After 12 hours of home cage fasting, blood for measurement of fasting insulin concentration is gently collected using a single cut at the tip of the tail. Meanwhile, fasting glucose concentrations (fasting glycemia) are measured from whole blood using a glucometer (Fig. 40 A).

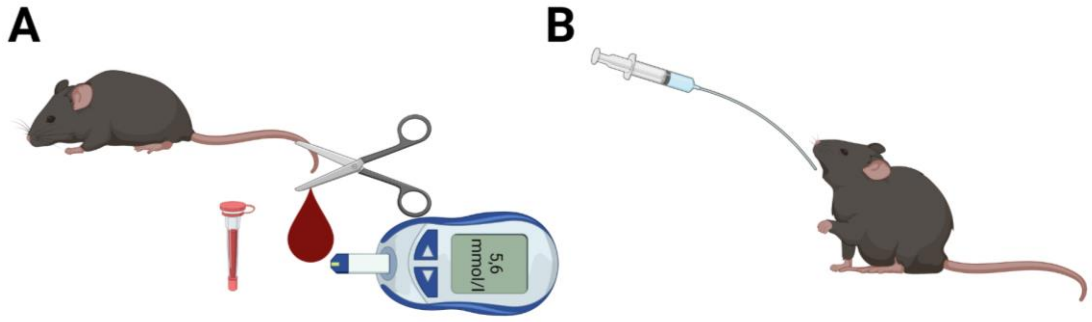


Fig. 40 A – Measuring of glycemia in experimental animals; B – Oral gavage method. Created with BioRender.com.

After measuring the fasting parameters from blood, the glucose solution in a dose of 2 g/kg of body weight is administered orally to each laboratory animal. For oral gavage, a special gavage needle is used. Animals are immobilized and a gavage needle is loaded into the stomach (Fig. 40 B). Glycemia is measured 15, 30, 60, 90, and 120 minutes after oral administration of glucose solution. The performance of the oral glucose tolerance test needs silent environmental conditions. A single cut at the tip of the tail is enough to collect all of the blood samples.

Insulin resistance in experimental conditions can be also detected using the calculations of indexes such as homeostatic model assessment for insulin resistance index (HOMA-IR) or the index of insulin sensitivity (QUIKI) can be calculated. Both indexes are calculated from fasting insulin concentrations in plasma, and from fasting glycemia in blood.

$$HOMA - IR = \frac{fasting\ insulin\ (\mu IU/l)\ x\ fasting\ glycemia\ (mmol/l)}{22.5}$$

$$QUIKI = \frac{1}{log\ (fasting\ insulin\ (\mu IU/ml\ )\ +\ log\ (fasting\ glycemia\ (mg/dl))}$$

9.3 Blood pressure measurement in laboratory rodents

At our institute, we have a so-called CODA® mouse and rat tail-cuff system that allows us to accurately measure the blood pressure of a single mouse or rat (Tab. 12). The CODA® tail-cuff blood pressure system utilizes volume pressure recording (VPR) sensor technology to measure the mouse or rat tail blood pressure non-invasively (Fig. 41). This method allows the measurement of six blood pressure parameters: systolic, diastolic and mean blood pressure, but also, heart rate, tail blood volume and blood flow.

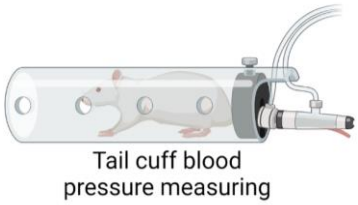


Fig. 41 Blood pressure measurement using the tail-cuff method. Created with BioRender.com.

The occlusion cuff is used to prevent blood flow to the tail. The second tail cuff, which has a sensor (VPR cuff), measures the physiological characteristics of the returned blood as the first tail cuff slowly discharges. The VPR cuff measures tail swelling as the blood flows back to it because blood flow causes pulsations in the arterial walls. Automatic systolic blood pressure monitoring begins as soon as the first manifestation of the tail swelling occurs. When tail swelling subsides, the diastolic blood pressure is automatically measured.

For the blood pressure measurements, animals need to be habituated to environmental conditions (light conditions and temperature of the testing room) and also for the restrainer tube (Fig. 41). This means that the animals have to undergo at least three blood pressure measurements on three consecutive days to reduce stress in animals and be able to measure reliable blood pressure values. Before testing the

room temperature needs to be set up (around 26 °C). Before setting up the tail cuffs on the animal tail, the temperature of the animal tail needs to be also measured. The appropriate tail temperature is 32 to 36 °C.

Protocol for blood pressure measurement:

1. Place the animal in a restrainer tube.
2. Measure the temperature of the tail.
3. Put the occlusive cuff as close as possible to the tail base. The occlusion cuff needs to be placed on the tail base without feeling resistance on the cuff. If you feel resistance the underlying artery and veins are pressed, and it results in unrealistic blood pressure values.
4. Next, place the VPR cuff on the tail of the animal. Again, pay attention to placing the VPR cuff without any resistance.
5. At last, follow the CODA system instruction for measuring the blood pressure of the animal.

Tab. 12 Reference values for physiological parameters of blood pressure measurements.

	Rat	**Mouse**
Heart rate	300-500 bpm / min	325-780 bpm / min
Systolic blood pressure	84-134 mmHg	113-147 mmHg
Diastolic blood pressure	60-90 mmHg	81-106 mmHg

9.4 Biochemical analysis of plasma samples

Besides obesity, insulin resistance, and hypertension, in patients with metabolic syndrome dyslipidemia, non-alcoholic fatty liver disease, hyperuricemia, and kidney damage is also present. In general, **dyslipidemia** is characterized by an increased concentration of triglycerides (hypertriglyceridemia), increased concentration of low-density lipoproteins (LDL), decreased concentration of high-density lipoproteins (HDL), and increased concentrations of total plasma cholesterol (tCHOL). The term **hyperuricemia** refers to a state in which concentrations of uric acid in plasma are higher than normal. **Non-alcoholic fatty liver disease** is characterized by increased

accumulation of triglycerides and cholesterol in liver cells (hepatocytes). This phenomenon leads to liver damage and failure, represented by increased concentrations of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP).

Hyperglycemia and high blood pressure linked to metabolic syndrome are related to impaired glomerular filtration in the kidneys. Creatinine (CREA) is excreted by the kidneys under normal conditions, but impaired glomerular filtration results in high plasma concentrations of CREA.

All aforementioned biochemical parameters can be determined in plasma. Plasma samples from patients, but also, rodents can be measured using an automatic biochemical analyzer (Biolis). Blood needs to be collected in heparin collection tubes and centrifuged at 1600G for 10 minutes at 4 °C. After centrifugation plasma is pipetted into 1.5 ml Eppendorf tubes in the volume of 0.5 ml (Fig. 42).

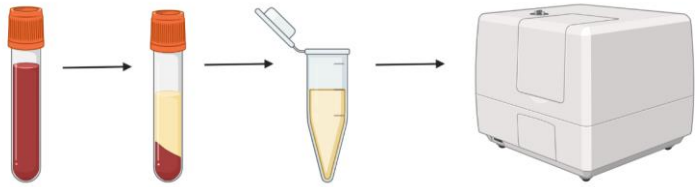


Fig. 42 Sample preparation for biochemical analysis of plasma using Biolis. Created with BioRender.com.

9.5 Urine collection in laboratory rodents (Alexandra Gaál Kovalčíková)

Metabolic cages are used for the collection of urine and feces, or for the measurement of animals' food and water intake. This device provides high separation efficiency of urine and feces (Fig. 43). According to general rules, urine should be collected in sufficient volume, without contamination, within the shortest time possible, and obtained without pain or distress. For the evaluation of kidney functions such as daily diuresis, creatinine clearance, or protein excretion, animals should be placed in metabolic cages for 24 hours. However, the disadvantage of 24-hour urine collection is that mice lose more than 10% of their body weight. Although standard rodent chow can be placed into cages, it is not recommended to use it due to possible contamination of urine by the chow. Therefore, the better option is to place mice into metabolic cages for 3 hours and then recalculate diuresis for 24 hours. An average diuresis during 3-hour collection in our laboratory conditions is 0.5 ml which is not much. Sucrose (5% or 10% solution) can be used instead of water to increase diuresis. Another problem is

the evaporation of urine. Inserting a 1.5 ml eppendorf tube with a conical bottom into the urine container reduces evaporation. Rats can be placed in cages for 24 hours if it is needed.

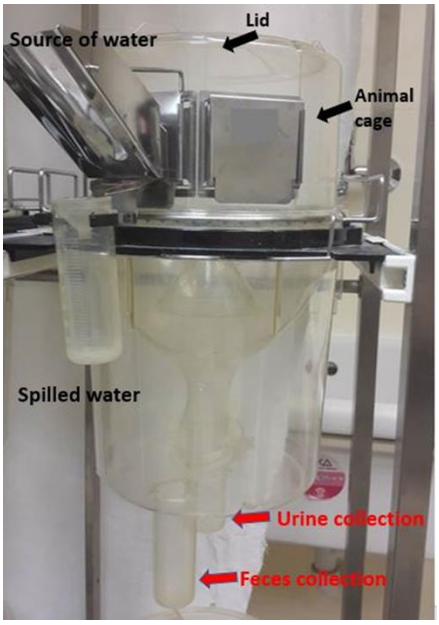


Fig. 43 Metabolic cage at laboratory of IMBM. Photo by Alexandra Gaál Kovalčíková.

9.6 Assessment of kidney functions

9.6.1 Creatinine

Assessment of creatinine in plasma and urine is an important laboratory method used for the evaluation of kidney functions. Creatinine is a low-molecular weight molecule that is spontaneously produced in skeletal muscles from its precursor creatine phosphate. Serum creatinine concentration is constant and depends on the portion of muscle mass, gender, age, and ethnicity. Additionally, creatinine is also influenced by more variable factors such as diet. Serum creatinine is used for the estimation of glomerular filtration rate (eGFR), which is accepted as the best overall index of kidney functions. Several formulas have been described to estimate GFR based on serum creatinine concentration and patient characteristics. Creatinine is excreted from the body through the kidneys into the urine. In addition to serum creatinine, urinary creatinine measurement is also of considerable diagnostic importance in order to calculate creatinine clearance, standardize proteinuria and albuminuria, and calculate fractional excretion of different solutes by kidneys.

Creatinine clearance rate (CrCl) approximates the calculation of GFR because creatinine is freely filtered in the glomerulus. However, it is also removed from the body by the process of tubular excretion, which causes an overestimation of GFR by approximately 10%. Nevertheless, CrCl assessment is accepted as a rapid and effective method for measuring GFR (Tab. 13).

$$Creatinine\ clearance = \frac{(urine\ creatinine\ (mg/dl) \times urine\ output\ (ml))}{(serum\ creatinine\ (mg/dl) \times collection\ time\ (min))}$$

Calculation for 24 hours collection = 1440 minutes

Tab. 13 Human reference values.

Serum creatinine	50-110 μmol / l
Urinary creatinine	7-20 mmol / 24 hours
Creatinine clearance	2 ml/s (1.3 - 2.8 ml / s); 120 ml / min

As mentioned above, for laboratory animals, especially mice that should not be placed in metabolic cages for 24 hours due to rapid decrease of the percentage of their body weight. 3-hour collection time is often used. In calculation, this collection time has to be taken into account. Moreover, creatinine clearance is re-calculated per 100 g of body weight. Mouse reference values of creatinine evaluated in our laboratory conditions are mentioned in Tab. 14.

Tab. 14 Reference values of creatinine in healthy mice measured in our laboratory conditions (might change according to strain of animals).

Serum creatinine	20-60 μmol / l
Urinary creatinine	0.1-2 μmol / 24 hours
Creatinine clearance	0.01-0.1 ml / min / 100 g

Measurement of creatinine using different variations of the Jaffé reaction or enzymatic methods is conventionally available, cheap, simple, and relatively reliable. So far, the most used method for the measurement of creatinine in plasma or urine in our laboratory is the Jaffe reaction. It is based on the reaction of creatinine with picric acid in an alkaline condition that results in a reddish complex. The intensity of the complex is proportional to creatinine concentration. However, picrate might also react with interfering substances such as proteins, glucose, ascorbic acid, or ketones which should be considered.

9.6.1.1 Measurement of creatinine in urine using the jaffe method

Protocol for the reagent preparation:

1. Prepare 0.2 M NaOH (molecular weight = 39.997) by its dissolving in distilled water. This solution is stable and could be stored at room temperature
2. Prepare 25 mM solution of picric acid (molecular weight = 229.104). Picric acid is slightly soluble in water (around 12 g / l). To prepare 1000 ml of this solution, dissolve 5.728 g of picric acid in 500 ml of water and fill up to 1000 ml. This solution is stable and can be stored at room temperature. Due to its explosive properties, the picric acid powder contains sizable water content (10 – 30 %). When weighing, try to take the powder from the bottom of the bottle where the chemical is relatively homogenous, not from the top where is the higher content of water.
3. For preparing a fresh reagent, mix NaOH and picric acid in a ratio 5:1. Twenty ml of reagent is needed per 96-well plate.

Table 15. Calibration curve for urinary creatinine measurement. Stock standard of creatinine: 1000 mg / dl.

Number	Concentration (mg/dl)	Standard (µl)	Water (µl)
1.	100	100 µl (from conc. 1000 mg / dl)	900 µl
2.	50	500 µl (from conc. 100 mg / dl)	500 µl
3.	30	600 µl (from conc. 50 mg / dl)	400 µl
4.	15	500 µl (from conc. 30 mg / dl)	500 µl
5.	7.5	500 µl (from conc. 15 mg / dl)	500 µl
6.	3.75	500 µl (from conc. 7.5 mg / dl)	500 µl
7.	1.875	500 µl (from conc. 3.75 mg / dl)	500 µl
8.	0	0 µl	1000 µl

Protocol for the standards preparation:

1. Prepare creatinine stock solution with the concentration of 1000 mg/dl
2. Prepare 8 eppendorf tubes. Label them from 1 to 8.
3. Add 900 µl of distilled water to tube 1. Add 100 µl of stock solution (1000 mg/dl) and mix properly.
4. Add 500 µl of distilled water to tube 2. Take 500 µL of the creatinine solution in tube 1 and add it to tube 2 and vortex properly.
5. Repeat dilution according to Tab. 15. Always add water (diluent) first.
6. Use water as a zero standard (blank).
7. Standards can be stored at -20 °C.

Procedure:

1. Firstly, turn on the computer and the spectrophotometer
2. Initialize the software Gen5 (Fig. 44) and set the absorbance at 492 nm

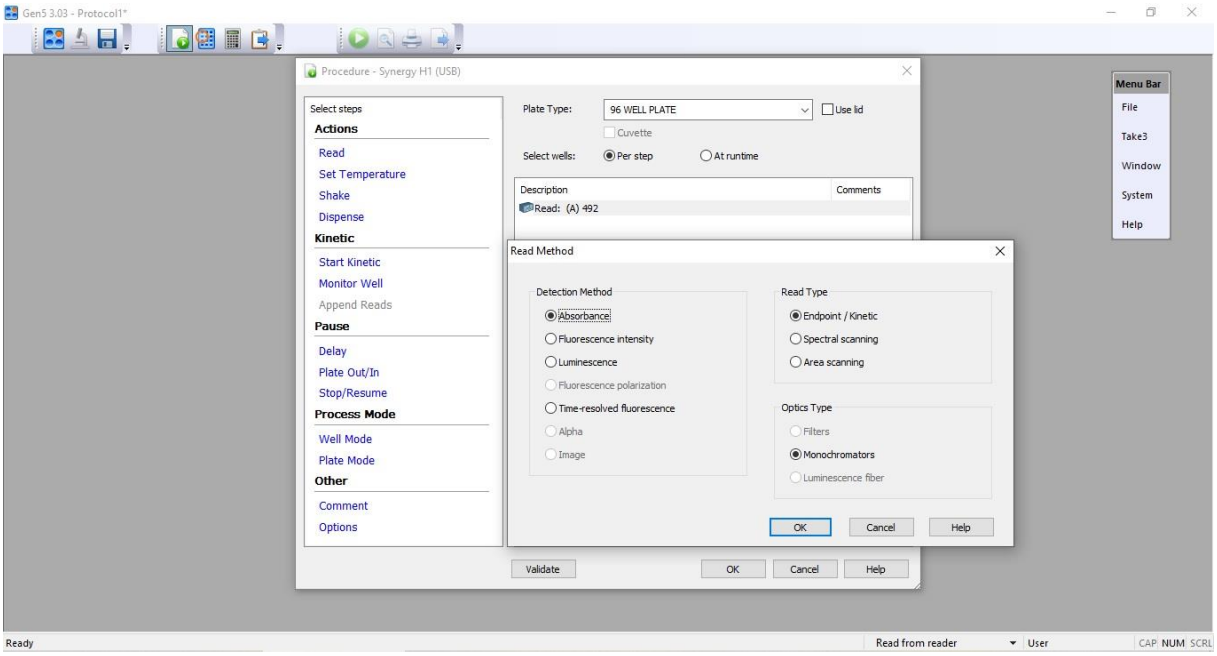


Fig. 44 Gen5 software. For the urinary creatinine measurement, reate a new protocol and set the absorbance at 492 nm, or open the existing protocol.

3. Pipette 10 µl of urine samples and 10 µl of standard (at least in duplicates, ideally in triplicates) to 96-well microplate. Pipette standards from the highest concentration to the lowest concentration. Use distilled water as a blank.
4. Add 200 µl of freshly prepared reagent into the samples and standards using a multichannel pipette
5. Measure absorbance after the 1st and 6th minutes.
6. Values after the 1st minute serve as blank.
7. In calculation, absorbance after the 1st minute is subtracted from the absorbance after the 6th minute. Calculate mean values of standards. Construct the calibration curve.
8. Calculate concentrations of samples according to the calibration curve (Fig. 45).
9. We use SI units for the expression of values. Therefore. conversion of mg / dl to µmol / l is needed. Conversion factor for creatinine is 88.42.

6th minute minus 1st minute									
Absorbance									Standards concentration
Samples					Standards		aver	aver minus blank	mg/dl
0,23	0,236	0,091	0,14	0,105	1,157	1,129	1,129	1,126	100
0,303	0,195	0,122	0,126	0,082	0,737	0,648	0,648	0,645	50
0,299	0,001	0,151	0,118	0,153	0,383	0,345	0,345	0,342	30
0,336	0,353	0,116	0,082	0,199	0,195	0,186	0,186	0,183	15
0,338	0,086	0,007	0,091	0,147	0,102	0,095	0,095	0,092	7,5
0,219	0,091	0,213	0,097	0,142	0,057	0,052	0,052	0,049	3,75
0,27	0,04	0,056	0,037	0,13	0,029	0,027	0,027	0,024	1,875
0,001	0,166	0,112	0,304	0,096	0,005	0,003	0,003	0,000	0

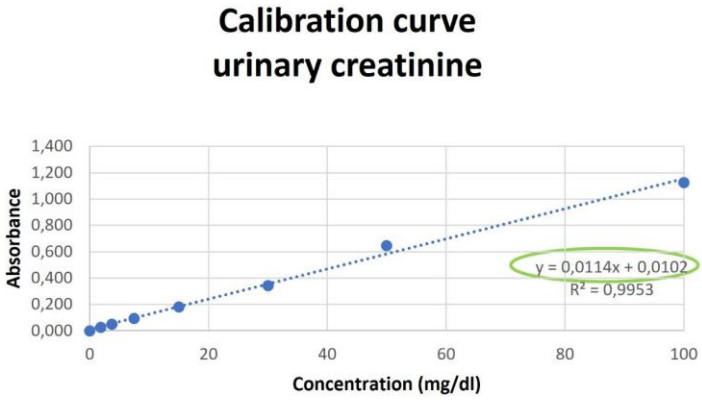


Fig. 45 Examples of raw absorbances of mouse urines and standards in urinary creatinine measurement. Subtract the absorbance measured after the 1st minute from the absorbance measured after the 6th minute. Calculate mean values of standards (from duplicates or triplicates, yellow highlighted). Construct the calibration curve using an average absorbance of standards and their corresponding concentration (red highlighted, y – means of background corrected absorbances, x – known concentration of standards). Add the linear trendline and display the equation (green highlighted). Calculate the concentrations of unknown samples according to the formula derived from equation.

9.6.1.2 Measurement of creatinine in plasma and serum

To minimize the volume of samples, creatinine in plasma or serum, especially in small laboratory animals, is usually measured using commercial spectrophotometric assay according to the manufacturer's protocol (Creatinine Serum Low Sample Volume; Urea Nitrogen Colorimetric Detection Kit. Arbor Assays, Ann Arbor, USA). This assay is based on the Jaffe reaction that is performed in 384-well plate format. In this assay, serum, EDTA and heparin plasma samples might be used. All blood samples should be centrifuged at 1600 g for 10 minutes. For the measurement, 15 µl of samples and standards are mixed with 15 µl of assay diluent and 60 µl of creatinine reagent provided by the manufacturer. Absorbance is measured at 490 nm after 1st and 30th minutes, 1st minute serving as the baseline.

Additionally, creatinine in plasma, serum, or in urine can be measured also using Biolis 24i Premium automated clinical analyzer (Tokyo Boeki Medical System Ltd., Tokyo, Japan). However, using the automated analyzer requires a higher volume of samples, which is related to "dead volume".

9.6.2 Proteinuria

Physiologically, human urine contains a small amount of proteins. Daily excretion of protein in the urine is 50-90 mg/24 hours, in physical activity up to 150 mg/24 hours. Physiological microalbuminuria ranges from 10 to 30 mg/24 hours. Proteinuria is defined as the presence of more than 150 mg of proteins in urine/24 hours, while albuminuria means more than 30 mg of albumin in urine/24 hours. Proteinuria and albuminuria assessment are commonly used for the detection of impaired kidney functions. Except for kidney disease (renal proteinuria), changes of hemodynamics (pre-renal proteinuria – pregnancy, dehydration, excessive exercise, fever, cold, hemolysis), or tumors and inflammation of lower urinary tract (post-renal proteinuria) could be associated with increased urinary proteins excretion. In laboratory animals (mainly rats), bigger concentration of proteins in urine is physiological. Physiological values of urinary proteins assessed in healthy mice in our laboratory conditions are mentioned in Tab. 16.

Tab. 16 Reference values of proteins and PCR in healthy mice measured in our laboratory conditions (might change according to strain of animals).

Urinary proteins	0.20 - 30 mg / 24 hours
PCR	900 - 9000 mg / g

Proteinuria is evaluated in preference in urine collected for 24 hours. In smaller laboratory animals such as mice, shorter periods (3 hours) could be used for urine collection. In calculation, the length of this period has to be taken into account. As mentioned above, except for glomerular filtration, excretion of protein into urine during the day is influenced also by external factors such as food composition or hydration. Therefore, urinary creatinine is used to standardize proteinuria and albuminuria. Thus, protein to creatinine ratio (PCR) and albumin to creatinine ratio (ACR) measurements can be performed in spot urine. Nevertheless, evaluation of PCR and ACR is an important indicator of kidney disease in humans, but also, in laboratory animals. Similar to creatinine

and protein excretion levels, the reference range of PCR in healthy mice was established in our conditions (Tab. 16).

9.6.2.1 Proteinuria determination using pyrogallol red method

Pyrogallol red with molybdate forms a red colored complex with an absorption maximum at 470 nm. In acidic conditions, fixation of this complex on proteins moves the absorption maximum to 600 nm. Intensity of blue-purple color is proportional to concentration of proteins.

Tab. 17 Stock solution of pyrogallol red 1.5 mM/l.

Pyrogallol red	60 mg
Methanol	100 ml

Tab. 18 Reagents needed for pyrogallol red solution.

Succinic acid (50 mM/l)	5.9 g	2.95 g
Sodium oxalate (1 mM/l)	0.134 g	0.067 g
Sodium benzoate (3 mM/l)	0.4323 g	0.21615 g
Disodium molybdate (40 µM/l)	0.00968 g	0.00484 g
Sodium dodecyl sulfate	0.025 g	0.0125 g
Brij 35	1 ml	500 µl
Add water to	1000 ml	500 ml

96 well plate – 30 ml

Protocol for reagent preparation:

1. Prepare 1.5 mM/l pyrogallol red stock solution (Tab. 17).

2. Dissolve succinic acid, sodium oxalate, sodium benzoate and disodium molybdate in 900 ml of water (400 ml in case of final volume 500 ml, Tab. 18).
3. Add 40 ml of stock solution of pyrogallol red /add 20 ml in case of final volume 500 ml.
4. Correct pH to 2.5 (using HCl).
5. Add sodium dodecyl sulphate and Brij 35. Dissolve granules of Brij 35 by heating before its adding into the reagent.
6. Fill up with a distilled water up to 1000/500 ml.
7. Store in the **dark** (using aluminum foil) at 4 °C (fridge).

Tab. 19 Calibration curve for proteins. Standard: BSA stock solution 10 mg/ml.

Number	Concentration (mg / ml)	BSA (mg / ml)	Water
1.	2	200 µl (from conc. 10 mg / ml)	800 µl
2.	1.5	750 µl (from conc. 2 mg / ml)	250 µl
3.	1	667 µl (from conc. 1.5 mg / ml)	333 µl
4.	0.75	750 µl (from conc. 1 mg / ml)	250 µl
5.	0.5	667 µl (from conc. 0.75 mg / ml)	333 µl
6.	0.25	500 µl (from conc. 0.5 mg / ml)	500 µl
7.	0.125	500 µl (from conc. 0.25 mg / ml)	500 µl
8.	0	0 µl	1000 µl

Protocol for the standards preparation:

1. Prepare bovine serum albumin (BSA) stock solution with the concentration of 10 mg/ml.
2. Prepare 8 eppendorf tubes. Label them from 1 to 8.
3. Add 800 µl of distilled water to tube 1. Add 200 µl of BSA stock solution (10 mg/ml) and mix properly.

4. Add 250 µl of distilled water to tube 2. Take 750 µL of the BSA solution in tube 1 and add it to tube 2, and vortex properly.
5. Repeat dilution according to Tab. 19.
6. Use water as a zero standard (blank).

Procedure:

1. Pre-heat the incubator to 37 °C.
2. Add 10 µl of urine samples and standards (preferentially in triplicates) into a 96-well microplate. Samples that are expected to contain high concentration of proteins (human samples from the patients with the kidney disease, samples from laboratory animals) have to be diluted in distilled water (usually 2-5 times). Otherwise, their absorbance is over the range of the calibration curve and cannot be used for the exact calculation of concentration. Use water as blank.
3. Add 300 µl of reagent into samples and standards. Do not forget to keep the reagent in the dark.
4. Incubate the plate for 15 minutes at 37 °C.
5. Turn on the computer and the spectrophotometer. Initialize the software Gen5 and set the absorbance at 595 nm (Fig. 46).
6. Read the absorbance at 595 nm.
7. Calculate the concentration of samples according to the calibration curve. **Dilution of samples has to be considered.**

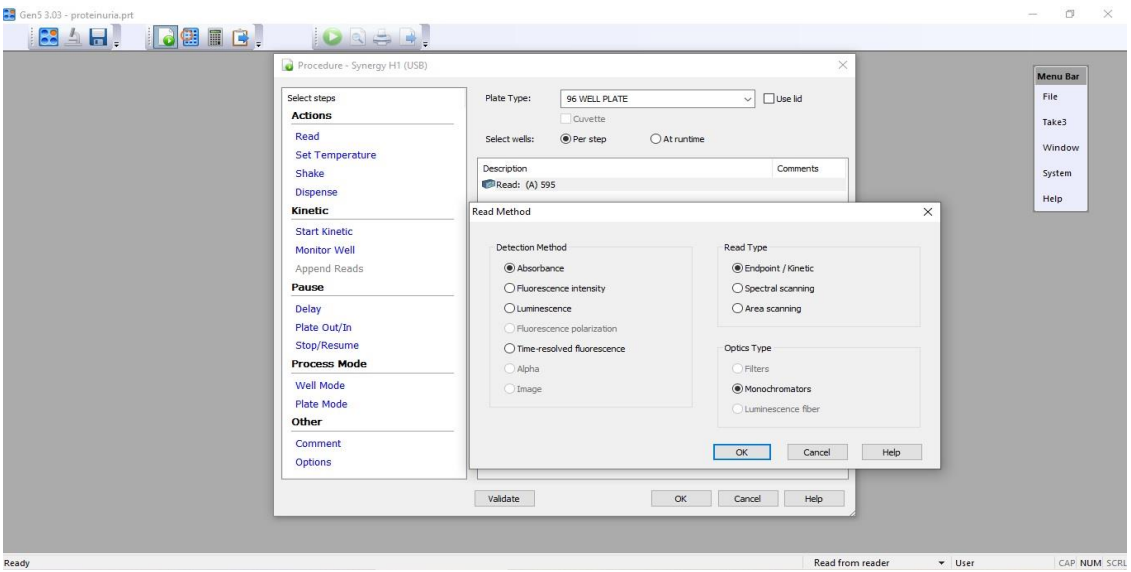


Fig. 46 Gen5 software. For a urinary protein measurement, create a new protocol and set the absorbance at 595 nm, or open the existing protocol.

Calculation of PCR:

Calculation of urinary proteins according to the constructed calibration curve gives the results in mg/ml that has to be converted to mg / l. Further, evaluation of urinary creatinine gives the results in mg/dl that has to be converted to mg / ml (g/l). PCR is calculated by the division of proteins in mg/l by creatinine concentration in g / l. However, PCR is often expressed in SI units – mg / mmol of creatinine. In that case, creatinine has to be converted from mg/dl to mmol/l, where **1 mg / dl of creatinine=88.42 µmol / l.**

$$\boldsymbol{PCR\ (mg/g) = \frac{urinary\ proteins\ (mg/l)}{urinary\ creatinine\ (g/l)}}\ \textbf{or}\ \boldsymbol{PCR\ (mg/mM) = \frac{urinary\ proteins\ (mg/l)}{urinary\ creatinine\ (mM/l)}}$$

10 In vivo imaging systems (Kristína Macáková)

10.1 IVIS® Spectrum In Vivo Imaging System

IVIS Spectrum – In Vivo Imaging System can be used to obtain the non-invasive imaging optical outcomes such as extent of inflammation, bone erosion or different bone deformities. IVIS method represents an in vivo imaging system which combines 2D and 3D optical tomography within one platform. IVIS offers the possibility of performing single-view 3D tomography for the fluorescent and bioluminescent reports.

Since the experimental animals should be anesthetized before every application. IVIS is connected with the isoflurane chamber which can be used as a separated single chamber, but it also has the possibility to communicate with the IVIS directly. Isoflurane chamber is filled out with the liquid isoflurane. Isoflurane chamber can be connected with the IVIS by turning on the switch situated on the isoflurane machine. Inside of the IVIS machine is a tubing system which can be placed to the rodent face. Tubing system is constructed for 4 experimental animals at one time. Inside tubing system with isoflurane is used for all the purposes of the IVIS, regarding the demanding stage of immobilization of rodents in all the different IVIS applications.

10.2 Micro-computed tomography

Micro-computed tomography is mostly used for the detailed study of the interior structures as bones or for the quantification of the extent of the swelling of paws within the live animals. As previously described, rodents before IVIS applications should be anesthetized. Based on the different duration of the exposure of the X-ray, eyes of rodents should be treated with the eye gel to prevent drying up of eyes. After rodents are fully anesthetized, they can be transferred from the anesthesia chamber to the inner part of IVIS. In the inner chamber of IVIS should be placed – specific perforative pad which is connected to the tubing system of anesthesia to let the isoflurane flow in during the whole period of the measurement.

To obtain a picture with the best resolution, only 2 rodents are placed in the holder with a tubing system; the holder on the pad is made for 4 rodents. After initial and necessary settings which are performed based on the factory manual, rodents are exposed to the X-ray. For security, meanwhile the X-ray is exciting, the attention light on the IVIS machine is turned on. After the exposure with the initial setting which also involves the mode of the picture which can be: fast, medium resolution, high resolution or high-resolution top, pictures are automatically saved to the prepared folder. Obtained picture will represent a 3D model of your rodent or the object of interest, for example, only a paw shown on Fig. 47. After saving and naming all the necessary folders and documents, rodents should be taken away from the IVIS chamber and treated with the post-anesthetized protocol.

Captured pictures have the format with the name *.dicom.* For the analysis you can use two different software. Living Image or RadiAnt DICOM Viewer. Both of them allow you to analyze *.dicom* file 3D structure or 2D structures from the different axis and plane, which for example allows you to measure the swelling of the soft tissue on rodent paws. Mentioned softwares also has the possibility to measure the bone density in the region of interest.

Fig. 47 Capture image with micro-computed tomography. Photo by Kristína Macáková.

10.3 IVIS® Bioluminescence Imaging System

IVIS can be also used for the detection of bioluminescence, which is a form of chemiluminescence, and it consists of the production of emission of light by living organisms. Bioluminescence also occurs in nature for example in fireflies and is mediated by the enzymes luciferin and luciferase. We are mostly using bioluminescence for the detection of the extent of inflammation. This means that the inflamed area is able to emit light. However, emission of light in rodents is not a spontaneous activity. Based on the non-spontaneous character, bioluminescence in rodents should be induced. With this

purpose we used a substance with the name luminol. Luminol is commercially supplied in the form of powder. Luminol is then prepared with PBS to the final concentration of 50 mg/ml. Dissolved luminol is filtered through the 0.2 μm filter. Because luminol is sensitive to the light, after dissolving the tube should be covered by the aluminum foil. Prepared luminol should be used the same day as it was prepared or then stored at -20 °C.

Prepared luminol at room temperature is applied intraperitoneally (i.p.) in the dosage of 200 mg / kg. After i.p. injection luminol should be absorbed in the body during the 10 minutes. During these 10 minutes rodents can be anesthetized in the chamber connected to the IVIS. Meanwhile mice are getting anesthetized. IVIS is set up for the bioluminescence detection based on the manufacturer protocol. One of the most important parts of the settings is time of the exposure, which is the time frame within the IVIS will detect the signal from the absorbed, excited signal from luminol of the inflamed area. Standard time for the exposition is 5 minutes. The time of exposure can be adjusted based on the expectation of inflammation, for the area with lower inflammation, longer time can be set up, but every project of the treatment could demand unique settings. Beside the different exposition times settings IVIS offers the possibility to use the application of software named Time Series Study which allows you to show the capture object in various time intervals. After choosing this application possibility is then essential to use the same time interval for every studied object. After naming and saving your acquired picture you are allowed to take away the experimental animal (animal inside of the IVIS) and treat it with post-anesthetized protocol. Another already anesthetized rodent can be placed inside of IVIS.

Saved pictures can be analyzed in the software Living Image. To quantify the bioluminescent signal, you have to choose the region of interest, for example, the inflamed paws shown on Fig. 48. After adjusting the area of interest, you can measure the zone. For the measurement of other regions of interest across various rodents you need to use the same size and shape of the zone of interest to avoid bias. After obtaining the final values of bioluminescence which comes in unit photons/s/cm2/sr and assigned to the specific groups of rodents, data can be analyzed.

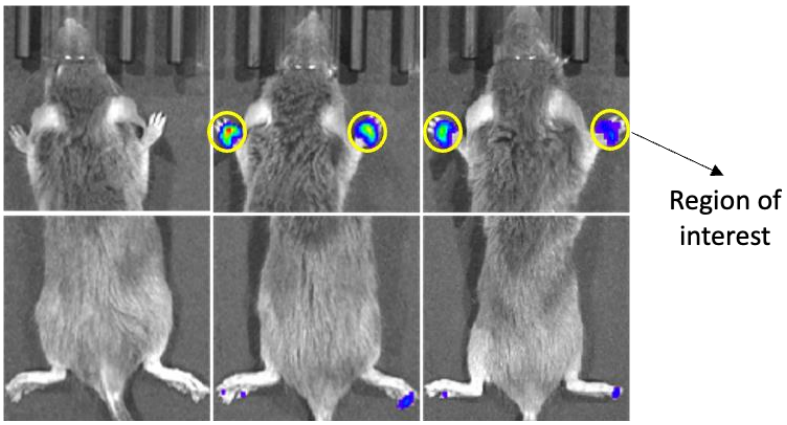


Fig. 48 Capture images after the bioluminescence detection together with the represented region of interest. Photo by Kristína Macáková.

10.4 Thermal imaging system

Another type of in vivo imaging method is to use a thermal camera. Thermal cameras can be used for controlling body temperature and the specific surface or tissue temperature in the red-blooded animals producing heat, e.g., rodents. Body temperature can be changed during the disease or thermal cameras can detect various local inflammation. The advantage of it is a non-invasive quantification of the extent of inflammation or the mentioned measurement of body temperature.

For the measurement of body or specific area temperatures it is crucial to establish the consistent room temperature to avoid the bias. Different or high temperatures of room can cause an inaccurate temperature measurement which is not caused by the vehicle or the treatment that you are performing with the selected group of rodents. Room temperature is normally set up and maintained at 25 °C. Rodents should be immobilized for thermal picture taking. The distance between the camera and rodents should be 20 cm.

For the following analysis at least 3 pictures, properly focused, should be taken. Per every saved thermal picture, the camera is also making and saving standard, normal pictures. Every picture has its specific number, so it is easy to organize the picture and to assign photographs of every rodent and to save to the prepared excel file.

Saved pictures are analyzed using FLIR Tools software (FLIR System, Inc.). In the analysis of pictures there are two approaches: to measure body temperature or to measure only one specific

selected zone, for example, temperature of paws (shown on Fig. 49). To determine paws temperature elliptical regions of interest should be selected. To final analysis, normalization of each paw divided through the mean of body temperature of the corresponding picture should be performed. To evaluate body temperature elliptical regions, encircling the entire body of rodents, excluding paws, must be done. Final temperature calculated by the software can be assigned to the studied group of rodents and statistically analyzed.

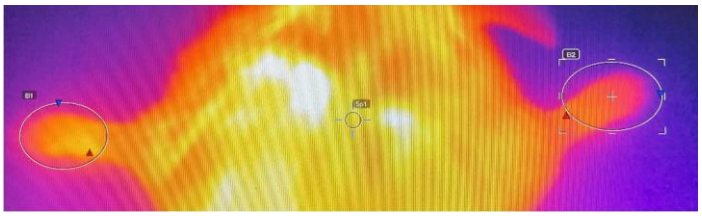


Fig. 49 Determining the area of interest in the analysis of thermal camera pictures with the purpose to determine temperature of paws. Photo by Kristína Macáková.

11 Polymerase chain reaction (PCR) method (Nikola Kováčová)

11.1 What is PCR?

The polymerase chain reaction (PCR) is an in vitro technique for producing numerous copies of a target DNA sequence. To put it simply, PCR copies DNA in a test tube just as replication occurs in living cells. DNA replication in a living cell is a sophisticated process that requires many different proteins and other molecules to occur precisely and with high fidelity. Compared to DNA replication, PCR requires commonly used reagents in the laboratory. But the principle is the same: After the unwinding of the double-stranded DNA, each parental strand is used as a template for the formation of a complementary 'daughter' strand. DNA polymerase, the enzyme that synthesizes DNA, needs a primer that is complementary to the template strand because DNA polymerase can incorporate new nucleotides only into an already existing nucleotide strand. Each nucleotide is then incorporated at the free 3′-OH end according to Watson-Crick base pairing rules using the parental strand that serves as a template.

The principle of the method was discovered in 1983 by biochemist Kary Mullis, who was awarded the Nobel Prize in Chemistry for his discovery. Since then, PCR has become an essential tool used in both scientific and clinical laboratories.

11.2 How does PCR work?

DNA amplification in the PCR reaction is a cyclic process. The reaction takes place in a thermocycler, which can increase or decrease the temperature of the reaction mixture within a few seconds. Changes in temperature affect the components of the reaction mixture which activity results in the amplification of the target DNA fragment (Fig. 50).

Each cycle consists of three basic steps:

- **Denaturation (95-98 °C)**
- **Annealing (50-60 °C)**

- **Polymerization (72 °C)**

The first step in the PCR reaction is denaturation. During denaturation, hydrogen bonds between base pairs are disrupted due to high temperature. The result is the formation of two single-stranded DNA chains that serve as a template. In the second step, the temperature is decreased, which allows the hybridization of primers on the complementary template DNA. Primers are oriented to opposite DNA template strands at positions defining the ends of the segment to be amplified. Hybridized primers form a signal for the polymerase, which starts by adding free complementary nucleotides to their 3'OH end. This step is called polymerization, and its beginning and end are bordered by forward and reverse primers.

These three steps, denaturation, annealing, and polymerization are cyclically repeated 20-30 times. The number of copies grows exponentially – even DNA fragments that were created in one cycle are used as a template in the next cycle. After n cycles, we get 2^n, and after 30 cycles 2^{30}, i,e, billions of fragments of the target DNA fragment (0.2-1 μg DNA).

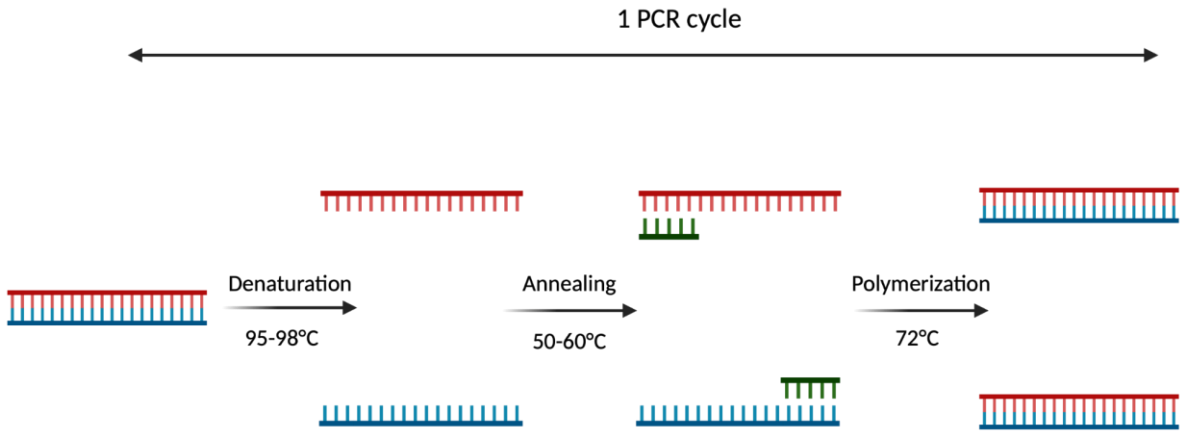


Fig. 50 Amplification of target DNA sequence.

11.3 Reagents

The PCR reaction includes DNA, enzyme (polymerase), primers (oligonucleotides), reaction buffer, and free nucleotides. Some reagents are mainly limited in the process such as polymerase, while others, like primers and nucleotides, are much more abundant.

11.3.1 Nucleotides

dNTP, or deoxynucleoside triphosphate, are the building blocks of DNA. There are 4 types of dNTP, and each type has a different nitrogenous base attached to the deoxyribose: adenine (dATP), cytosine (dCTP), guanine (dGTP), and thymine (dTTP). The one-letter term (A, C, G, T) is used when writing the sequence of nucleotides in the analyzed DNA molecule. Nitrogenous bases are formed by one or two structural rings, and they are divided into pyrimidines, which include a pyrimidine ring attached to the deoxyribose, and purine, which also contains a pyrimidine ring, which is fused to an imidazole ring.

Free nucleotides are monomers that connect to each other in a polynucleotide structure and form a polymer – DNA strand. The nucleotides that form the DNA strand are linked by a phosphodiester bond between the phosphate groups of the nucleotides. With the help of the polymerase enzyme, these monomers are incorporated into the newly synthesized strand according to the complementary template strand. Complementarity means that in DNA, adenine preferentially pairs with thymine from the opposite strand of the double helix, and guanine with cytosine. Complementary base pairs are joined by hydrogen bonds – there are two hydrogen bonds between A and T and there are 3 hydrogen bonds between G and C. The recommended concentration in the PCR reaction is 200 μM of each of the deoxynucleotides.

Free nucleotides are sensitive to defrosting. Therefore, it is not recommended to work with the entire volume – it is appropriate to prepare aliquots from the total volume and work with them instead. Furthermore, it is optimal to prepare a container with ice or a cooling rack for test tubes before working with dNTP. The use of ice or a cooling rack will prevent the degradation of dNTPs.

Confusing nomenclature

The difference between a base, nucleoside, deoxynucleoside triphosphate, and nucleotide may be sometimes confusing. Since the terms were mentioned above, a brief explanation is presented in the following section.

A nucleotide has 3 components: a nitrogenous base (adenine, guanine, cytosine, thymine), a sugar component, and a phosphoric acid residue. If all these components are present, we talk about a nucleotide. In the absence of phosphate residues, we talk about a nucleoside. If the hydroxyl group (-OH) is absent at the 2' end of the sugar component, we speak of a deoxynucleotide (deoxy- means the absence of a hydroxyl group). The hydroxyl group at the 3' end plays an important role in nucleotide incorporation during polymerization. During DNA polymerization, a reaction occurs between the 3'-OH group of one nucleotide and the phosphate group of the new dNTP. In the absence of a hydroxyl

group at the 3' end, the polymerization reaction will be stopped. This means that deoxynucleotides are the substrate for DNA synthesis. To provide information about the number of phosphate residues, deoxynucleotides can be called as deoxynucleoside (mono, di, or tri) phosphates. Thus, deoxynucleoside monophosphate, deoxynucleoside diphosphate and deoxynucleoside triphosphate are nucleotides.

11.3.2 Primers

As mentioned earlier, DNA polymerase is not able to synthesize the complementary strand of DNA without a primer. Primer initiates DNA replication in vivo and in vitro – the polymerase starts replication at the 3'-OH end of the primer and copies the opposite strand. But further, we will only talk about the primer used in laboratory conditions. Primers are short synthetic oligonucleotides, usually about 16-25 nucleotides long and are complementary to the 3' ends of both target DNA strands. The primers flank the target sequence we want to amplify and create a short double-stranded region that serves as a signal for the polymerase to begin DNA synthesis. In PCR, one pair of primers are (usually) needed – forward and reverse. The forward primer is complementary to the DNA strand in the 5'-3' direction. The DNA strand that runs from the 5'-3' end is coding, sense, and non-template strand. The reverse primer is complementary to the DNA strand in the 3'-5' direction. The DNA strand that runs from the 3'-5' end is a non-coding, anti-sense, and template strand.

There are several rules to follow when selecting or designing primers for the successful amplification of the target sequence:

- Primers that are used in one PCR reaction must have the same or approximately the same (+/- 5 °C) melting temperature. The melting temperature (Tm) is the temperature at which exactly half of the primers are in single-stranded form. The ideal melting temperature of the primers is 50-60 °C and is calculated according to the formula Tm = 4*([nC] + [nG]) + 2*([nA] + [nT].
- The primer sequence must be unique. It is important that the primers anneal only to a specific region of the template sequence and not to other sequences with partial complementarity. Therefore, when designing primers, it is necessary to verify their specificity by comparing the proposed sequence of the primer with sequences available in public databases.
- The specific binding of primers is closely related to the annealing temperature, which is approximately 3-5 times lower than the melting temperature of the primers. If the annealing temperature of the primers is too low, the primers will anneal to non-specific regions on the

template DNA resulting in non-specific PCR products. On the other hand, too high annealing temperature can lead to a low yield of PCR products.

- An even distribution of nucleotides is recommended, while no more than 4 identical nucleotides should follow one after the other.
- The content of cytosine and guanine bases should be 40 - 60 %.
- Furthermore, it is necessary to consider that complementary base pairs can be paired within the same primer, as well as forward and reverse primers can pair with each other, resulting in a non-specific PCR product. Therefore, it is necessary to design primers with the lowest possible self-complementarity.
- The number of primers also affects the result of the PCR reaction. If there are many primers in the reaction, the probability of forming primer dimers increases. Conversely, if there are few primers in the reaction, the result will be a low yield and sensitivity of the PCR reaction. That's why in PCR, equal concentrations of both primers are (usually) used, with the recommended final concentration of each primer 0.1-0.5 μM. In general, a concentration of 0.2 μM is suitable for most reactions, but in some cases optimization of the primer concentration is necessary.

There are several publicly available websites that we can use to design primers, for example, Primer3 or PerlPrimer. However, primer design is not the only option. Primer sequences can be obtained from scientific publications, or we can use commercial primers.

11.3.3 Template

A template in a PCR reaction can be any source of DNA, such as genomic DNA (gDNA), complementary DNA (cDNA), or plasmid DNA. Template DNA is a double-stranded molecule that contains the target sequence and is isolated from the biological materials (organs, blood, body fluids, etc.) of an organism (human, animal, plants, bacteria, viruses). Methods for isolating template DNA differ based on the source of the DNA (for example, we obtain DNA from tissue differently than from blood). The final product of the PCR reaction depends on the quality, purity, and integrity of the template DNA molecule. It is important that the template DNA does not contain polymerase inhibitors such as detergents, organic solvents, proteases, nucleases, or excess RNA, for example, after isolation, residues of phenol, ethanol, EDTA, SDS, and chloroform may remain in the sample which can affect the PCR reaction, the output of which is a negative or false negative result. We will deal with PCR

inhibitors in a separate section. The result of the PCR reaction is also affected by the amount and concentration of template DNA that enters the reaction and differs from the source from which it was isolated. It is generally recommended to add 50-500 ng to the reaction. If we add a large amount of template DNA to the reaction, it may happen that all primers will bind to it at the same time and the reaction will be inhibited.

11.3.4 Polymerase

DNA polymerases are capable of DNA synthesis in the 5'-3' direction. They are not capable of de novo synthesis and need an already existing strand with a free 3'OH. We add thermostable polymerase to the PCR reaction, which is active even at high temperatures used in PCR. The most used in PCR is Taq polymerase, which was isolated from the thermostable bacteria Thermophilus aquaticus. The optimal reaction temperature of this enzyme is 74 ˚C and Taq polymerase remains functional after incubation at 95 ˚C for more than 40 min. It has a high rate of dNTP incorporation – it works at a rate of 100-150 nucleotides per second. On the other hand, it does not have 3'-5' exonuclease activity (or so-called proofreading activity). Proofreading activity means that enzyme can "check its work" during DNA synthesis. Since Taq polymerase does not have this activity, it is prone to introducing base-pairs errors during synthesis. Although the error rate of Taq polymerase is low, there are some applications that require the accuracy of the enzyme. For example, if the next application of the amplified DNA is sequencing and thus reading or determining the primary DNA sequence. The recommended concentration of the enzyme in the PCR reaction is 25 units/1 ml, i.e., 1.25 units per 50 μl reaction. An excess of enzymes can increase the formation of non-specific products.

11.3.5 Reaction buffer

The buffer provides a suitable environment for primer annealing and DNA polymerase activity. The buffer can be supplied together with the polymerase when it is optimized for the reactions. However, there are situations when we have to set the optimal concentration of magnesium ions ourselves. Magnesium ions are cofactors of DNA polymerase. The concentration of magnesium cations affects

the PCR reaction: as their concentration increases, the yield of PCR increases, and at the same time the specificity of the reaction decreases. The concentration of the MgCl2 should be in the range of 1.5–5.0 mM.

11.4 PCR inhibitors

PCR inhibitors are all substances that harm the PCR reaction. They may originate from the sample such as blood, tissues or may be introduced into the sample during its processing or during the extraction of nucleic acid. Inhibition of the PCR reaction subsequently leads to a decrease in sensitivity, the formation of non-specific products, or false negative results. Inhibitors can disrupt the individual steps of the PCR reaction. For example, the template can be modified or degraded by nucleases, the hybridization of primers to the template can be disrupted, or proteases or other detergents present in the reaction can directly inhibit the polymerase. Substances that are important for proper cell lysis and purification of nucleic acids, such as EDTA, SDS, ethanol, isopropyl alcohol, phenol, and salts (e.g., sodium chloride or potassium chloride) may contribute to reducing the efficiency of the PCR reaction. Therefore, it is very important to choose a suitable method for sample processing and nucleic acid extraction.

Practical tip: Before the blood collection, it is necessary to think about the types of analysis we want to use it for. Anticoagulants such as EDTA can inhibit PCR. Therefore, if we know that the sample will be used for PCR analysis, we cannot take blood into blood collection tubes with EDTA as an anticoagulant. EDTA is a chelating agent that may deplete magnesium ions and thus inhibit DNA polymerase activity.

Since PCR is sensitive to inhibitors whose effects can lead to non-specific or false-negative results, it is recommended to use standardized controls.

11.5 Positive and negative controls

PCR is a very sensitive method. As mentioned above, a very small amount of input sample is required for the PCR reaction. However, the sensitivity of the method also brings disadvantages: the method is sensitive to the presence of contamination. The use of controls ensures the accuracy of the assay. The

negative control is a master mix with almost all reagents (dNTP, polymerase, primers, reaction buffer), but we do not add a template to the reaction. Instead of the template, we add deionized water. With a negative control, we check for potential contamination. If everything is fine, this sample should not be amplified (because it does not contain a template). If the PCR product is present in the negative control after the end of the reaction, the reaction is contaminated. Contamination means that we have unwanted DNA in one of the PCR components (we may have contaminated buffers, dNTPs, primers, or water) or we introduced contamination into the reaction by non-sterile pipetting. The false positive result causes the unreliability of the positive reaction even for reactions with a present DNA sample. Therefore, it is necessary to repeat the reaction, replace the components of the reaction, or tighten up the work to achieve a perfectly DNA-free environment. In addition to the negative control, it is recommended to use a positive control as well. A positive control is, simply put, a sample of "what it should look like". The positive control contains all reagents including template DNA whose amplification result is known. If there is no DNA amplification in the positive control, we cannot rely on the results of the other reactions for example, when all samples come out negative. The cause of a false negative result may be inhibited polymerase activity or one of the reagents being added in an insufficient amount.

11.6 Real-time PCR

Real-time PCR, also known as quantitative PCR, is a method in which we can also determine the exact amount of the DNA template (quantity) in addition to its presence (quality). Quantification is to find out your original amount of sample – how much sample was in the tube when you pushed the RUN at the thermocycler. Real-time PCR is a fluorescent version of standard PCR and relies on all the reagents of a standard PCR reaction, such as template, polymerase, primers. dNTPs, and reaction buffer. However, in addition to these components of the reaction, another component is also necessary – fluorescent signal source, because fluorescence is used to detect the increase in DNA fragments over time during the reaction and not after its termination, as is the case with endpoint PCR. The increase in fluorescence is directly proportional to the increase in the amplified product during the PCR. As a source of the fluorescence signal, we can use an intercalation fluorescent dye (SYBR Green I or its derivatives such as EvaGreen) or a fluorescent probe (Molecular Beacons, TaqMan). SYBR Green is usually a component of the master mix for the real-time PCR reaction. SYBR Green only binds to double-stranded DNA – it does not bind during the denaturation and annealing step, but only after the end of the polymerization, when we already have a completed amplicon (synthesized double-stranded

PCR product). The disadvantage of SYBR Green is the non-specific binding of the intercalating fluorescent dye to dsDNA. The dye detects all dsDNA, so even primer dimers can be quantified. We can overcome this problem by verifying the specificity after the reaction is over. We can determine whether the fluorescence signal results from the formation of the specific PCR product or a non-specific product or primer dimer. The analysis is based on the melting temperature of the PCR product. The melting temperature depends on the length and sequence composition (the content of GC pairs) of the DNA fragments. At the end of the reaction, we have a double-stranded PCR product to which SYBR Green is attached. Subsequently, there is an increase in temperature and a decrease in fluorescence (because SYBR Green binds to dsDNA and not to ssDNA). Non-specific, specific PCR products and primer dimers can therefore be distinguished by the given analysis based on different melting temperatures and changes in fluorescence. Another disadvantage is that we cannot use SYBR Green for multiplex PCR (we would not be able to distinguish several samples by one color). At the same time, the advantages of SYBR Green or other intercalating fluorescent dyes include, for example, their sensitivity. Since its binding to dsDNA is non-specific, one amplicon can contain more than one bound dye = the fluorescence signal will be more intense.

Another option is to use a fluorescent probe. A fluorescent probe is used for specific detection – it is sequence-specific and therefore does not incorporate into a random place on dsDNA as in the case of SYBR Green. The probe contains two fluorescent dyes: a reporter dye on the 5’ end and a quencher dye on the 3’ end. When they are in close proximity the reporter dye is not able to emit photons because the quencher dye blocks its activity and, in that case, no fluorescence is measured. When part of the reaction mixture, they are in close proximity the entire time. Even after binding the probe to a specific DNA region, no photons are emitted because the probe is not long enough, and the reporter dye and quencher dye are still close to each other. During primer extension, the polymerase reaches the site of the template where the reporter dye is bound and removes it by its 5'-3' exonuclease activity – the reporter dye moves away from the quencher dye and the reporter emits fluorescence. Finally, the polymerase reaches the position of the template where the quencher dye is and thus removes the entire probe. The advantage of fluorescent probes is that they are specific. Probes are designed together with primers and are complementary to the target sequence of the DNA fragment. The probe binds to the template DNA only if it is complementary to the sequence, which results in high specificity of the reaction because there is a low probability that non-specific hybridization of the primers and the probe would occur. Among the other advantages of the fluorescent probe is its applicability to simultaneous amplification and detection of different target sequences in one reaction (in one test tube).

After each cycle of the real-time PCR reaction, the intensity of fluorescence is measured and the data from all the cycles are used to construct an amplification plot. The amplification plot is dependent on the detected fluorescence level on the y-axis and the time or cycle on the x-axis (Fig. 51). During the first cycles of the reaction (approx. up to 15 cycles), the fluorescence is low. Low fluorescence

represents background fluorescence or baseline. The critical point is the cycle when the threshold cycle (Ct) is exceeded. It is generally accepted that the lower the Ct value, the more copies of the template DNA are at the beginning of the reaction. Ct values are the basic result of the reaction, and they can be exported from the cycler software and work with them further (e.g., in Excel).

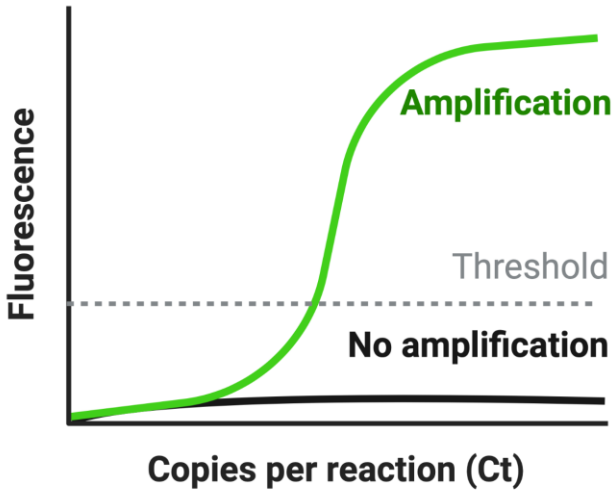


Fig. 51 Amplification plot

The real-time PCR method is used to monitor gene expression. When monitoring gene expression, messenger RNA (mRNA) is analyzed. All cells have uniform genetic information, but not all genes are expressed in all cells. Likewise, in different developmental stages, cells express different genes. Therefore, we monitor gene expression changes at the mRNA level. However, in PCR it is impossible to analyze RNA. Hence, it is necessary to transcribe RNA into cDNA. This process is called reverse transcription. The enzyme reverse transcriptase is necessary for this process, which is supplied as a separate enzyme. The transcription itself takes place in a separate reaction.

11.7 Protocol for conducting PCR method

1. Preparation of the reaction mixture:

The first step in the preparation of the master mix is calculating the volumes of the individual reagents. An explanatory table of the basic calculations is presented below (the initial concentrations of the given components may vary depending on the reagents used; the Tab. 20 serves as a template and the values serve as a guide).

Tab. 20 Calculation of the amount of reagents per reaction.

Components	Stock concentration	Volume for 1 reaction	Final concentration	Volume for (n + 1) reactions
Reaction buffer	5 x	x µl	1 x	
dNTP	10 mM	x µl	200 µM	
Forward primer	10 µM	x µl	1 µM	
Reverse primer	10 µM	x µl	1 µM	
Taq Polymerase	5U/µl	x µl	1.25 U	
DNA template		10 µl		
Water				
Total:		50 µl		

Always start by calculating the appropriate amount per reaction. Each ingredient must have the right concentration. An equation can be used for calculation: $C_1*V_1 = C_2*V_2$. Here is an example of calculations of individual reaction components with a considered final volume of 50 µl:

- Reaction buffer: (5 x)*(x µl of the buffer from stock) = (1 x)*(50 µl)

The resulting volume of 1x concentrated reaction buffer per reaction is 10 µl.

- dNTP: (10 mM)*(x µl from stock) = (200 µM)*(50 µl)

Do not forget to convert units! The resulting volume of dNTP per reaction is 1 µl.

- Primers: (10 µM)*(x µl from stock) = (1 µM)*(50 µl)

The resulting volume of primer per reaction is 5 µl (5 µl of forward an 5 µl of reverse primer).

- Taq polymerase: 1.25 U*(1 µl/5 U)

The resulting volume of Taq polymerase per reaction is 0.25 µl.

- Water: 50 µl – (10 µl buffer + 1 µl dNTPs + 5 µl forward primer + 5 µl reverse primer + 0.25 µl Taq polymerase) – 10 µl template = 18.75 µl of water per reaction

The volume of water per reaction is calculated at the end when the volumes of the other components per reaction are known.

After calculating the volumes per reaction, the volumes for the master mix are calculated (Tab. 21). It is recommended to count with one extra reaction because there may be losses (for example during pipetting). Thus, there may not be enough master mix volume for the last PCR tubes. In the given example, there are 4 reactions (n=4), but the calculated volumes per reaction are multiplied by 5 (4 + 1 extra reaction).

The total volume of the master mix is 40 µl * 5 = 200 µl. The calculated volumes of the components (except for the DNA template) are then pipetted into the test tube. Mastermix is prepared in a PCR box, which ensures a DNA-free environment (the possibility of contamination by unwanted DNA is reduced). The preparation of one master mix is useful to avoid technical variability. After adding all the components, the master mix is mixed by short vortexing to make the mixture homogeneous. The prepared master mix is then divided into 4 PCR tubes and the template is added to each tube individually. This was a concrete example for better understanding. Of course, the amount of reaction may vary. Therefore, it is key to mention that apart from separate plastic PCR tubes, there are also so-called strips – 8 plastic PCR tubes connected into one strip. Another alternative is a PCR plate (8x12) with 96 wells. The type of plastic tube is selected based on the number of reactions. It is crucial to adapt the reaction volume to the chosen type of test tube. With separate tubes, the volume may be 100 µl and with 96 well plates, it may be up to 20 µl.

Tab. 21 Final calculation of the amount of reagents.

Components	Stock concentration	Volume for 1 reaction	Final concentration	Volume for 5 reactions
Reaction buffer	5 x	10 µl	1 x	50 µl
dNTP	10 mM	1 µl	200 µM	5 µl
Forward primer	10 µM	5 µl	1 µM	25 µl
Reverse primer	10 µM	5 µl	1 µM	25 µl
Taq Polymerase	5U/µl	0.25 µl	1.25 U	1.25 µl
DNA template		10 µl		
Water				93.75 µl
Total:		50 µl		

Thermocycler:

The prepared reaction is then inserted into the wells of the thermoblock (Fig. 52). The pre-set program in the thermocycler software is selected or a new program is created depending on the reaction. Temperature, length of each step, and the number of cycles is set.

Fig. 52 PCR Thermocyclers at IMBM. Photo by Nikola Kováčová.

2. Analysis of the PCR product:

Gel electrophoresis is used to analyze the PCR product by dividing the fragments in an agarose gel, which is placed horizontally in a buffer solution. DNA fragments of the same length move in the gel at the same speed and form a band in the gel. DNA has a negative charge, and therefore. DNA fragments move from the cathode (-) to the anode (+) in the electric field. The gel is prepared from agarose which forms a network. Moreover, there are pores in the network that allow the movement of DNA molecules during electrophoresis. The size of the pores depends on the concentration of agarose. The higher the concentration of agarose, the more suitable the gel is for dividing smaller molecules.

Agarose gel is prepared by dissolving agarose in the TBE (Tris-borate EDTA) or TAE (Tris-acetate EDTA) buffer. The same buffer is further used in the apparatus, in which the gel is placed, and the separation takes place. The gel must be fully immersed in the buffer, otherwise, the electrophoresis will not proceed correctly. To ensure an equal dissolution of the agarose in the buffer, the mixture can be boiled. After the dissolution, an intercalation dye (e.g., GoldView) is added to the mixture. Such mixture is poured into a container with an electrophoresis comb that creates wells in the gel. The solidified gel can be used for electrophoresis.

A 1 % or 1.5 % gel is usually used to visualize PCR products. Before application. it is necessary to mix the PCR product with the application buffer, which is colored and makes it easier to pipet into the agarose gel. Moreover, the application buffer allows us to follow the movement of DNA fragments in the gel. A DNA standard is usually pipetted into the first well in the gel. A DNA standard is a set of

DNA fragments whose size is known. The PCR product is pipetted into the other wells. Electrophoresis takes place in an electric field at 5-10V/cm. After electrophoresis, the DNA is isolated under UV light. PCR product size is determined by using a DNA standard.

12. The isolation of DNA and RNA (Ľubica Janovičová)

12.1 DNA isolation

DNA isolation is one of the most common methods used in molecular biology in both research and clinical laboratories. Isolated DNA is suitable for subsequent analysis in different ways depending on the quantity and experimental procedures that follow. A large number of protocols have been developed that vary in the quantity of DNA that can be isolated, properties of isolated DNA, and purity. Commonly used methods are DNA isolation with phenol and isolation using silica membrane.

12.1.1 Phenol:chloroform DNA isolation

The isolation of DNA using phenol is based on sample lysis and subsequent denaturation of proteins in samples using phenol. During centrifugation, DNA remains in the liquid phase while denatured proteins are pelleted. DNA from the liquid phase is precipitated using ethanol and washed in the final steps, DNA isolated using phenol contains both, very short and long fragments of DNA. This isolation is prone to contamination with proteins, phenol, and ethanol. Additional wash steps of pellets help to remove these contaminants.

Procedure:

1. Add an equal volume of the phenol:chloroform:isoamyl alcohol mixture to the volume of the sample (mixed in a ratio of 25:24:1). Mix by vortexing for about 15 seconds.
2. Centrifuge the mixture for 5 minutes at 16 000 × g. After centrifugation, carefully manipulate with samples. Phases form in the sample mixture. The upper phase is aqueous and contains DNA since it is soluble in water. The lower phase contains phenol and proteins. Pipette the aqueous phase into the clean tubes without touching the lower phase.
3. Add 2.5 volume of 100% ethanol and 0.5 volume of 7.5M ammonium acetate to the aqueous phase. This precipitates the DNA.

4. Allow the mixture to precipitate for several hours at -20 °C in the freezer (ideally overnight). Then centrifuge the mixture in a cooled centrifuge (4 °C) for 30 minutes at 16 000 × g. A pellet of DNA forms at the bottom of the tube.
5. Carefully remove the supernatant using a pipette. Add 150 µL of 70 % ethanol to the pellet. Centrifuge in a cooled centrifuge (4 °C) for 5 minutes at 16 000 × g.
6. Carefully discard the supernatant by pipetting and let the pellet dry at room temperature for 10 minutes.
7. Dissolve the pellet in TE buffer or water.
8. Check the purity and amount of isolated DNA with a Nanodrop.

12.1.2 Column DNA isolation methods

The principle of column isolation is the binding of the DNA present in the solution to the column and the elution of the DNA from the column. This is possible due to the presence of salts and the change in pH in the reaction. A disadvantage of column kits is that very short DNA fragments are lost as they do not bind the silica membrane as effectively as longer fragments. There are many commercial DNA isolation kits available. It is essential to select the right kit according to the type and amount of sample and the number of samples from which DNA will be isolated. Some samples may be more difficult to isolate since they either contain inhibitors that inhibit the isolation or need longer lysis time e.g., tougher tissues such as muscles or bones. The specialized kits for isolation e.g., bacterial DNA or stool DNA can be used.

In principle, DNA isolation using an isolation kit is very simple and the protocol by the manufacturer should be followed. After lysis, a sample containing DNA is spun down to allow DNA to bind to the silica membrane. Proteins, lipids, and impurities do not bind the silica membrane and are removed in the washing steps. DNA is released from the silica membrane by incubation with TE solution (pH 9.0) or with deionized water and by final centrifugation. The isolated DNA must be stored at -20 °C until further analyses. The purity and concentration of isolated DNA in samples need to be measured with a Nanodrop.

12.2 RNA isolation

The methodology of RNA isolation also depends on the purpose for which this RNA is needed or how it will be analyzed. Most often, the goal of RNA isolation is the analysis of gene expression in tissues and cells and many other applications. The instability of RNA is emphasized in all the manuals. Due to the instability of RNA rapid enzymatic reverse transcription of RNA into cDNA is recommended. Moreover, the presence of RNases in samples, the enzymes that break down the RNA presents a problem in RNA analysis. To eliminate the activity of RNases, diethyl pyrocarbonate can be used for isolation solutions. Use of special tubes for the collection of samples intended for RNA analysis such as RNAlater can be employed. Generally, fast processing of samples either by keeping them on the ice at all times or freezing them in liquid nitrogen is enough to acquire good quality RNA from isolation.

The isolation procedure itself is similar to DNA isolation. Tissues or cells are lysed, proteins removed and RNA is precipitated and washed to remove contaminants. Guanidine isothiocyanate together with phenol denature proteins in samples. This is followed by ethanol precipitation. There are two isolation options – using a commercial column kit or using Trizol (or other commercially available variants). The advantage of RNA isolation columns is that isolated RNA has higher purity in comparison to Trizol. However, Trizol can be used to isolate RNA from large tissue samples and isolates all RNA found in the sample. Additionally, Trizol can be used to isolate not only RNA but DNA and proteins too.

The result of RNA isolation is a mixture of different RNAs. From tissues, this is predominantly ribosomal RNA. In electrophoresis, there are usually two bands that represent 18S and 28S ribosomal subunits. The degradation of RNA can be assessed when looking at these two peaks of RNA. The RNA integrity number – RIN, is calculated and used as a measure of RNA quality. The absence of peaks is often caused by RNA degradation before, during, or after isolation. After each isolation, it is important to do several analyzes of quantity and quality. Without it, it is not possible to estimate the amount of template needed for rtPCR. If the RNA analyses are not working troubleshooting is needed. Contamination of RNA can be solved by purification on a column with a silica membrane. RNA degradation cannot be fixed, therefore, isolated RNA or tissue aliquots should be prepared for these cases.

13 Neutrophils (Emil Bečka)

Neutrophils are the most abundant white blood cells in humans and represent approximately 59% of nucleated cells in blood and bone marrow. Neutrophils belong to a family of polymorphonuclear cells or PMNs, due to their lobulated nucleus in matured form. Matured neutrophils typically last in circulation for 6-8 hours and after that go back to the bone marrow, where they end their journey under physiological conditions. Those that migrate to the tissues during inflammation can however prolong their viability to 2-3 days. Neutrophils participate in the innate immune response, and they are the first immune cells that respond to the presence of a pathogen and migrate to the affected tissue from blood in large numbers. During ongoing infection, the lifespan of neutrophils is prolonged and granulopoiesis in bone marrow is increased.

In response to microbes, neutrophils employ different strategies aimed at their elimination. The first described response is phagocytosis, where neutrophils engulf the microorganisms and lyse them in their phagolysosomes using proteolytic enzymes. Neutrophils can also eject these enzymes to the extracellular space from the intracellular storage granules in a phenomenon called degranulation, In both mechanisms, reactive oxygen (ROS) and nitrogen species are produced that in addition serve in the coordination of these and other neutrophil responses.

13.1 Neutrophil extracellular traps (NETs)

Next to phagocytosis and degranulation, neutrophils can in response to pathogens also produce neutrophil extracellular traps (NETs). NETs formation is a process in which NADPH oxidase drives the production of superoxide that subsequently activates neutrophil elastase and myeloperoxidase that are released from their granules. These enzymes then cleave the nuclear membrane and nucleic proteins responsible for packaging and condensation of DNA to chromatin. This process results in the unfolding of chromatin in the cell, finally leading to the rupture of the cytoplasmic membrane and the release of neutrophil DNA decorated with cytoplasmic and granular proteins into the extracellular space.

Up to date, NETs have been described to fulfill these roles:

- immobilization of microbes and prevention of their dissemination
- pathogen killing due to the presence of antimicrobial peptides and proteases
- stimulation of autocrine and paracrine cell signalization – swarming of other neutrophils to the site of activation and activation of other immune cells, which are sensitive to the presence of NETs
- cleavage of proinflammatory cytokines and attenuation of the inflammation process through the action of proteases

On the other hand, excessive NETs formation has be implicated in several pathologies:

- sepsis – NETs are generated as a result of excessive neutrophil activation caused by cytokine storm, which in turn generates more NETs in a vicious cycle. These NETs then damage endothelial cells causing endothelial dysfunction,
- thrombosis – NETs serve as a scaffold that induces platelet adhesion, followed by their aggregation and red blood cell recruitment. The whole thrombus then forms together with fibrinogen and fibronectin,
- atherosclerosis – NETs activate macrophages that produce proinflammatory cytokines and damage the arterial smooth muscle by histone – DNA complex,
- cancer – NETs proteases can activate proliferation of dormant malignant cells and in addition assist in metastasis formation by working as a literal net for circulating tumor cells,
- autoimmune diseases – components of NETs such as citrullinated histones or DNA-histone complexes are a target of autoantibodies in autoimmune diseases such as lupus erythematosus and rheumatoid arthritis and NETs are therefore considered to play a significant role in their etiology and progression.

13.1.1 NETs structure

Besides common structural proteins, neutrophils contain granules filled with hydrolytic enzymes and antimicrobial proteins and peptides. The majority of these proteins have a positive charge, which allows for the formation of complexes with negatively charged DNA. This interaction is enabled during the process of NETosis when chromatin unwinds inside the cell and granule membranes have already been ruptured. The following proteins are the most represented in NETs: myeloperoxidase,

neutrophil elastase, cathepsin G, proteinase 3, metalloproteinase-9, cathelicidin, lactoferrin, tertiary gelatinase, histones, Lysozyme C and calcium-binding proteins S100.

13.1.2 Induction of NETs formation

NETs formation was reported to be induced by a variety of stimuli, ranging from pathogens such as bacteria, viruses, fungi, and parasites to sterile stimuli such as mitochondria, platelets, immune complexes, and urea crystals as shown in the Fig. 53. *In vivo*, this process usually happens also in the presence of increased pro-inflammatory mediators, such as interleukin-8, tumor necrosis factor, or lipopolysaccharide. During in vitro experiments, the organic compound phorbol 12-myristate 13-acetate (PMA) is broadly used as a positive control for the induction of NETs.

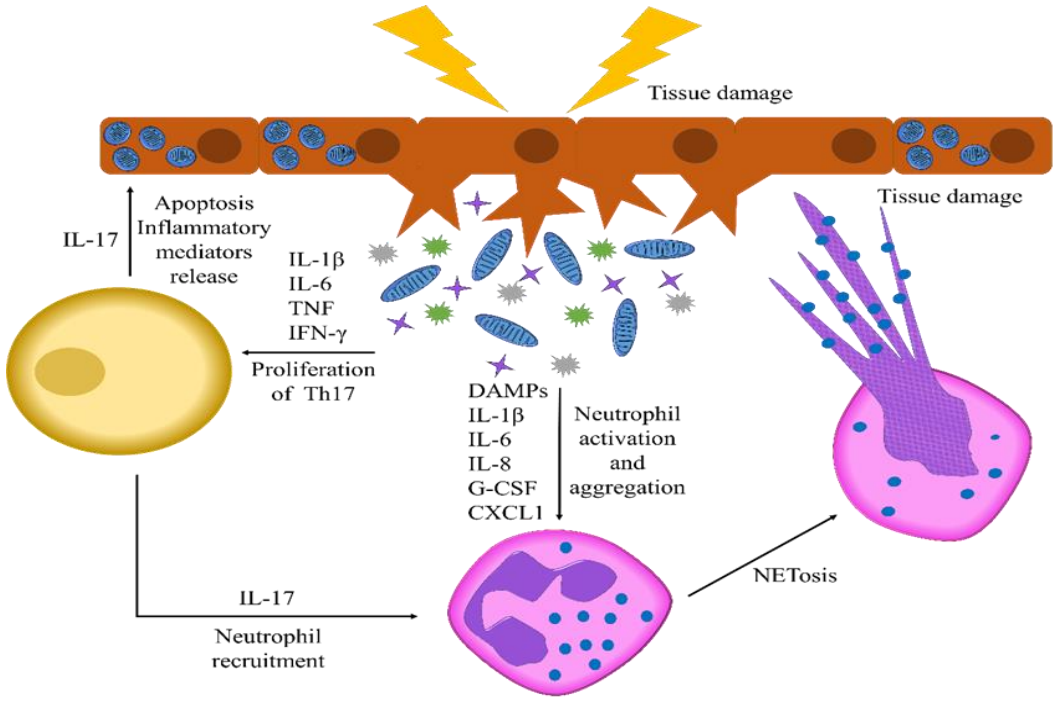


Fig. 53 Cytokine storm activates neutrophils. After tissue injury, cracked cells eject their content of cytosol into the extracellular matrix, where most of them are recognized as DAMPs. Alive but damaged cells produce cytokines as altering signal for immune innate and adaptive immune system. Abbreviations: DAMPs, damage-associated molecular patterns; G-CSF, granulocyte-colony stimulating factor; IFN, interferon; IL, interleukins; TNF, tumor necrosis factor; XCXL, C-X-C motif chemokine ligand.

13.1.3 NETs formation mechanism

In contrast to biological inductors, which usually trigger NETosis via their corresponding receptors, induction of NETs by PMA is well described. PMA stimulates protein kinase C, which is an upstream mediator of NADPH oxidase activation. The active NADPH oxidase generates ROS from the molecular oxygen in the cell. These ROS, typically superoxide, have a double effect. First, they penetrate the granule membranes and leak in. In granules, ROS activate myeloperoxidase and neutrophil elastase, which are necessary for the rupture of the nuclear envelope and decondensation of chromatin. Meanwhile, calcium influx occurs in neutrophils as a result of their activation. Calcium cations are co-factors for peptidyl arginine deiminase 4, which modifies arginine to citrulline in nuclear histones. This allows for an elimination of the positive charge of histones, weakening the DNA-histone interaction, and decondensation of chromatin. After leaking from the nucleus, DNA is mixed with granular and cytoplasmic proteins. The rupture of both cytoplasmic and nuclear membrane during NETosis is facilitated by neutrophil elastase-activated gasdermin D, which forms pores in the membrane.

13.2 Practical neutrophil research

Even though the knowledge in the field of neutrophil biology is rapidly expanding, there are still many gaps to fill and processes to understand. In the following pages, we will describe basic methods for neutrophil isolation as well as an assessment of their function.

13.2.1 Isolation of neutrophils from venous blood

In normal conditions, 4 000-11 000 white blood cells are present in one microliter of circulating blood. Neutrophils typically represent about 55-70% of all leukocytes and can be isolated via different methods, with gradient isolation being one of the most common. Using saccharide-based solutions with defined density, it is possible to divide individual leukocyte populations. For neutrophils, commercially available solutions such as polymorphprep yield very pure (≥ 85 %

neutrophils/leukocytes) and vital (≥ 95 % of live cells) neutrophils after centrifugation and supporting purification steps.

Material

1. Blood vial with anticoagulant agent + blood sampling kit: optimal isolation of neutrophils requires heparinized blood, but a good alternative is also EDTA or sodium citrate.
2. Dextran: 3 % Dextran-500 (average molecular weight 200 000-500 000) dissolved in sterile and endotoxin free 0.9 % NaCl. It can be prepared as 30 g/l in saline.
3. Solution Ficoll-Hypaque: it is commercially available or can be prepared as a mixture of Ficoll 400 and isopaque to a final density of 1.077 g/ml.
4. Sterile deionized water and NaCl: for the next purification of neutrophils solutions of 1.8 %. 1.6 %. 0.45 %, and 0.2 % NaCl should be prepared.
5. Sterile cell medium without endotoxins (RPMI/HBSS) without Ca2 + and Mg2 +
6. Polymorphprep.

13.2.2 Isolation of human neutrophils

1. If plasma is needed, human whole blood in a heparin/EDTA tube can be centrifuged at 220 × g for 10 minutes, at room temperature (RT).

2. In a 15 ml tube, add PMN isolation medium Polymorphprep (5 ml) on the bottom of the tube and overlay it with the blood of the same volume (5 ml) and centrifuge at 550 × g for 30 minutes. RT, with brakes set to 1 on the oscillating rotor.

3. To harvest neutrophils, collect the third layer from the gradient with a Pasteur pipette (the first layer is plasma, the second layer is mostly peripheral blood mononuclear cells and the fourth is erythrocytes as shown in Fig. 54).

4. In a 50 ml tube, mix the PMN layer at a 1:1 ratio with 0.45 % NaCl, and wait for 5 minutes at RT to adjust for different osmolarity.

5. Fill the tube with RPMI or PBS (without adding fetal bovine serum), and centrifuge at 400 × g, for 10 minutes, at 4 °C.

6. After centrifugation discards the supernatant.
Tip: Before discarding the supernatant, prepare a 1000 microliter pipette with a sterile tip for fast resuspending of the pellet in the next step.

7. You can skip steps 7-9 if the pellet is white or slightly pink. For red blood cell lysis, gently mix the pellet with 20 ml of ice-cold 0.2 % NaCl for 20-30 sec. and immediately add 20 ml of ice-cold 1.6 % of NaCl (which makes for a final 0.9 % solution of NaCl).
   Tip: First, add 5-10 ml of 0.2% NaCl to resuspend the pellet properly, then add the remaining volume.
8. Centrifuge the tube at 400 × g, for 10 minutes, at 4 ℃.
9. Discard supernatant again.
10. Resuspend the pellet in a corresponding working solution required for your experiment (RPMI, HBSS, ... + 10% FBS) and let chill on ice for at least 15 minutes.
11. Count the isolated cells (Luna, Burker chamber, flow cytometer).

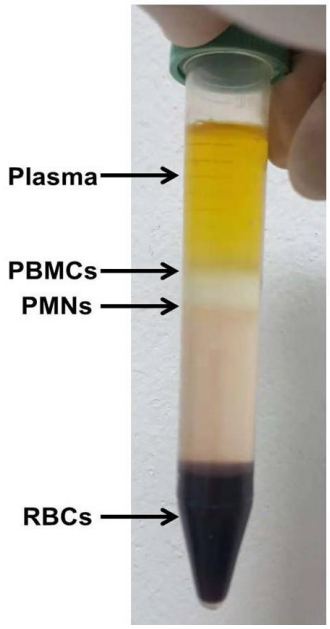


Fig. 54 Separated blood cells after gradient centrifugation. PBMCs – peripheral blood mononuclear cells. PMNs – polymorphonuclear cells. RBCs – erythrocytes. Photo by Emil Bečka.

13.2.3 Isolation of the neutrophils from the mouse bone marrow

Blood sampling from rodents is a common method, but the amount of blood is not sufficient for the isolation of neutrophils. The bone marrow is the organ where blood cells are produced and also serves as the storage of leukocytes in case of need. The isolation from bone marrow results in a higher yield than would be possible from the blood, but it must be noted that these cells may contain a significant portion of neutrophils that are yet not fully matured.

Prepare in advance: Ice, napkins, tweezers, Pasteur pipettes, Histopaque, and Percoll warm to RT, 25G needle, 10 ml syringe, ice-cold saline, Petri dish, 15 & 50 ml tubes, 40um cell strainer, ice-cold 0.2% & 1.6% NaCl.

- Solution A = RPMI + 10% FBS, 1% Penicillin/Streptomycin + 2mM EDTA
- RPMI (ice-cold) 500 ml
- 10% FBS (ice-cold) 56 ml
- 0.5M EDTA → 2 mM 2.25 ml (±)
- PCN-SM → 1 % 5.6 ml
- Solution B = RPMI + 10% FBS

1. Collect mouse bones, clean them from the muscle with a napkin and wash them in saline in a Petri dish/store them until the next procedure.
2. Pierce (or cut if it is not possible to pierce) both epiphysis of all long bones with the 25G needle and wash the bone marrow out with ice-cold solution A in a 10 ml syringe. One bone requires 1 syringe and a change of the needle after each mouse is necessary. Collect the washed bone marrow to the 50 ml tube and fill with solution A.
3. Centrifuge the tube at 450 × g, for 10 min, at 4 °C.
4. Discard the supernatant and lyse the pellet in 20 ml of ice-cold 0.2 % NaCl (20 sec), then add 20 ml of ice-cold 1.6 % – you need to use the pipet to break down the pellet.
5. Repeat centrifugation according to step #3.
6. Resuspend cells with 1 ml of solution B & filter through a 40 μm cell strainer (1 for 1 mouse), and collect the supernatant with a 1000 ul pipette from the strainer.
7. In a 15 ml tube prepare 3 ml of 81% Histopaque, overlay with 3 ml of 55% Percoll, and overlay with 1 ml of cell suspension.
8. Centrifuge the tube using an oscillating rotor at 800 × g, for 35 min at RT with brakes set to 1.
9. Collect the neutrophil layer with a Pasteur pipette into a 50 ml tube. Hold the centrifuged tube against the light/window for precise recognition of the layer.
10. Wash the collected neutrophils with solution A with centrifugation as in step #3.
11. Resuspend the cells in 1 ml of solution B.

Cell counting

1. Resuspend the final pellet in 1 ml of cell medium. For the quantification of cells, dilute the 10 microliters from the cell suspension in 90 microlitres of PBS.
2. For cell staining and to check the vitality of cells, mix 10 microlitres of diluted cell solution with 10 microlitres of Trypan blue. Trypan blue stains only the membrane of the live cells, but stains the whole content of dead cells.
   Tip: Use a piece of parafilm or the lid of an already used centrifuge tube for mixing the cell suspension and Trypan blue.
3. Pipette a few microliters under the cover glass slide until the Burker chamber is filled with the solution.
4. Under the microscope, find the square shape as is imaged in Fig. 55.
5. Count the cells in the big square and also count the cells, which are situated on the borders of 2/4 of the border lines of the big square.
6. One big square has a volume of 0.1 microliters. Count the three big squares and do the average of the cell number. The final concentration of cells needs to be set as the number of cells in one milliliter. In our procedure, we must count with the volume of the big square and use dilutions:

$$concentration\ [cells/ml]\ =\ 10\,000 * 2 * 10$$

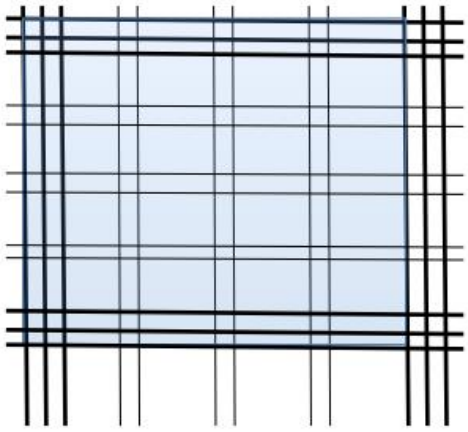


Fig. 55 Pattern visible in Burker chamber. The counting area of the big square is marked light blue.

13.2.4 Isolation of NETs

Prepare in advance: Sterile RPMI, neutrophils in concentration 2 million/ml at least 5 ml for one reaction, PMA, Petri dishes, DNase stock solution.

1. Isolate neutrophils and prepare a solution of 5 ml RPMI without FBS with 10 million neutrophils (2 million/ml). If you plan to isolate more NETs, along with the neutrophil count, the medium volume must be increased so the concentration stays the same.
2. Each batch of neutrophils must be incubated in tissue culture Petri dishes.
3. Use 400 nM of PMA (from a 40 μM stock, add 5 μl into 5ml), and incubate for 3 hours at 37 °C. 5 % CO2.
4. After incubation, pipette the first 3 ml of RPMI medium into a 15 ml falcon tube. Use the remaining 2 ml of RPMI to wash the surface of the dish, and use forceful pipetting so the NETs detach better. Use a cell scraper to separate the NETs from the surface better if needed. Add the 2 ml into the falcon tube.
5. Homogenize them with a hand sonicator. TIP: for increased quantities of isolated NETs, DNase can be applied. This is however not applicable when You want to analyze the nuclease resistance of NETs. For this procedure, apply 2 U/μl of DNase I (from the 2000 U stock mix 5 μl with 15 μl of RDD buffer), and incubate for 20 min at 37 ºC. 5% CO_2. Do NOT exceed the 20 min mark, because the DNase will destroy the NETs completely. Be careful with the DNase, it is sensitive to mechanical shock and will be rendered ineffective.
6. Centrifuge the supernatant at 400 × g, for 10 min, and the clumped NETs, and dead cells will pellet, leaving only NET-rich supernatant.
7. Centrifuge at 20 000 × g for 10 minutes at RT, to pellet the NETs from the supernatant.

13.2.4.1 Boyden chemotaxis assay

Prepare in advance: Neutrophil suspension in concentration 1.4 million cells/milliliter. N-Formylmethionyl-leucyl-phenylalanine (fMLF) stock solution, E. coli solution with OD 0.1 in RPMI. LC medium (10 μg/ml Hoechst 33342, 400 nM Sytox™ green in RPMI + 1% FBS), Boyden chamber.

1. Dilute the neutrophil suspension with a fresh LC staining medium at RT in a ratio of 1:1 to reach a final concentration of 0.7 million cells/ml and incubate the cells at 37 °C, 5 % CO_2 for 15 minutes.

2. During staining, fill the bottom well with 150 microliters of desired stimuli, such as 200 nM fMLF or E. coli set at OD 0.1, or recalculate it to your desired multiplicity of infection (MOI). Use at least two wells for one stimulus as the duplicate, for negative control use the cell medium in which the neutrophils and stimuli are dissolved.
3. Apply the upper part of the Boyden chamber.
4. After the incubation of neutrophils with fluorescence dyes is finished, pipette 75 microliters of suspension to the upper part of the apparatus and incubate it at 37 °C, 5 % CO_2 for 90 minutes. TIP: for murine neutrophils, time should be increased to 2 hours.
5. After the incubation, transfer the supernatant from the bottom part to the clean 96-tissue culture plate with a multi-pipette.
6. Centrifuge the well at 400 × g for 5 minutes and scan the plate with The Cytation 7 live imaging system or count via other cell counting methods. The neutrophils during incubation should go through the pores and stay in the bottom part. These neutrophils are stained blue in the case they are still intact and green, when they undergo cell death.

13.2.4.2 Ex vivo live NETosis assay

Prepare in advance: Neutrophil suspension in concentration 0.4 million cells/milliliter. PMA stock solution, E. coli solution with OD 0.1 in RPMI, LC medium (10 μg/ml Hoechst 33342, 400 nM Sytox™ green in RPMI + 1% FBS), 96-tissue culture plate.

1. Dilute the neutrophil suspension with a fresh LC staining medium at RT in a ratio of 1:1 to reach a final concentration of 0.2 million cells/ml and incubate the cells at 37 °C, 5% CO_2 for 15 minutes.
2. During staining, prepare the stimuli (PMA, bacteria, mitochondria) that should be 2x concentrated. For example, to have a final concentration of 100 nM of PMA, prepare it at the concentration of 200 nM. Pipette 100 microliters to the wells. Use at least two wells for one stimulus as the duplicate, for negative control use the cell medium, in which the neutrophils and stimuli are dissolved.
3. After the incubation, add 100 microliters of neutrophil suspension to each well with treatment.
4. Centrifuge the plate at 250 × g for 2 minutes at RT.
5. Insert the plate into the 37 °C pre-heated chamber of Cytation 7 live cell imaging system and scan of wells every 20 minutes for 180 minutes with magnification 10x. TIP: a simplified guide for setting up the protocol with a comment on the analysis philosophy can be found on the computer belonging to the Cytation 7 live cell imager).

6. NETs formation is evaluated as an area under the curve of all NETs across all time points (green fluorescent signal) normalized to the number of cells (blue fluorescent signal) at time zero and recalculated to % of NETotic neutrophils.

14 Extracellular vesicles (Alexandra Gaál Kovalčíková)

Extracellular vesicles (EVs) are membrane vesicles released by every prokaryotic cell, but also by cells of higher eukaryotes and plants. The importance of EVs probably lies in the ability to transmit information to other cells and thereby influence the function of the receiving cell. Signals mediated by EVs can be carried by different categories of biomolecules – proteins, lipids, nucleic acids and carbohydrates – this packet of information can be sent by the original cells in multiple places simultaneously.

Accumulating data indicate that EV content, size, and composition are highly heterogeneous and dynamic and depend on cell source, state, and environmental conditions. At least 3 main subgroups of EVs have been defined previously:

(a) apoptotic bodies,

(b) cellular microparticles/microvesicles/ectosomes (MVs),

(c) exosomes.

Apoptotic bodies are released directly from the plasma membrane during apoptosis. MVs include vesicles of various sizes that are formed directly from the plasma membrane during cell life. Finally, exosomes are intraluminal vesicles contained first in the MVB inside the cell and are then released into the extracellular environment after MVB fusion with the plasma membrane.

This nomenclature is currently developing since there exists a considerable overlap among groups, Therefore, it is possible that you experience the different terminology, such as small or large extracellular vesicles or even other names based on the origin of vesicles.

Extracellular vesicles could be isolated from extracellular space by various methods based on literature. You can find several chosen protocols below.

14.1 Protocol for the isolation of exosomes from plasma

1. Take blood into heparin tubes – 3 × 10 ml.

2. Centrifuge the collected blood at 1 600 g, 10 minutes, 4 °C; (pellet = apoptotic bodies).

3. Pipette the supernatant into 1.5 ml tubes and centrifuge at 16 000 g, 30 minutes, 4 °C; (pellet = MV).

4. Take the supernatant and aliquot it using a syringe into sealable centrifuge tubes (Polypropylene Quick Seal Centrifuge tubes, Beckman Coulter, Inc., Brea, CA, USA) and seal them.

• 2.5 ml of plasma into 1 tube and top up with PBS.

5. Ultracentrifuge in a fixed rotor 50.2 Ti at 100 000 g, 90 minutes, 4 °C. For this type of tubes, it is essential to use metal lids during ultracentrifugation according to the manufacturer's rules.

6. Collect the pellet – cut off the top of the tubes, remove part of the supernatant with a syringe, cut the tube in half, pour off the supernatant, collect the pellet by resuspending it in PBS – 3 × 25 ul. If anything floats at the bottom before pouring, it is a "cap" – also collect with a pipette.

7. Prepare the density gradient for ultracentrifugation:

- Working solution (Tab. 22, Tab. 23)

Tab. 22 Chemicals for preparation of a working solution.

2.5 M sucrose	5 ml
1 M Tris	600 ul
0.5 M EDTA	3 ml
dH_2O	41.4 ml

- Homogenizing medium

Tab. 23 Chemicals for preparation of a homogenizing medium.

2.5 M sucrose	5 ml
1 M Tris	100 ul
0.5 M EDTA	500 ul
dH_2O	44.4 ml

- To prepare a 50% OptiPrep solution. the working solution must be mixed with the commercial OptiPrep preparation in a ratio of 1:5.
- Density gradient:

Tab. 24 Density gradient layers.

	50% Optiprep	**Homogenizing medium**
12%	2.4 ml	7.6 ml
18%	3.6 ml	6.4 ml
24%	4.8 ml	5.2 ml
30%	6 ml	4 ml
36%	7.2 ml	2.8 ml

8. Pipette the sample into ultra-clear tubes (Beckman Coulter™ Ultra-Clear Centrifuge Tubes) – exosomes to the bottom and then the density gradient (Tab. 24):

- each gradient layer in a volume of 2.5 ml
- fill the top with PBS to approximately 3 mm from the surface

9. Ultracentrifuge in swinging rotor SW41 – 120 000 g, 15 hours, 4 °C.

10. Take the top 8 ml with a pipette and pipet into a sealing tube as in step 4. Add PBS.

11. Ultracentrifuge in a fixed rotor 50.2 Ti. 120 000 g, 4 hours, 4 °C. For this type of tubes, it is essential to use metal lids during ultracentrifugation.

12. Collect the resulting pellet in PBS, in a similar way as mentioned in step 6.

14.2 Protocol for isolation of exosomes from cell media

- aliquot the entire bottle of FBS into the largest possible centrifuge tubes – adapt the size to the size of the rotor.
- ultracentrifuge for 18 hours, 120 000 × g; depending on the type of tubes, we will use lids.
- in a sterile box, cut off the tops of the tubes and use a large syringe to aspirate all the supernatant, which we store in a sterile bottle.
- we filter the entire volume through a 0.22 μm filter, which we often change due to clogging (the filter still captures excess vesicles).
- in this way we obtain FBS without vesicles, which we add to the cell media and culture in the classic way.
- we store the obtained FBS at -20 °C.
- for 500 ml of cell medium, we use 50 ml of FBS (final concentration 10%).

1. After culturing the cells, collect the cell medium in sterile centrifuge tubes with a conical bottom.

2. Centrifuge the collected cell medium at 300g, 10 minutes, 4 °C (removal of cell remnants).

3. Filter the supernatant through a 0.22 μm filter.

4. Aliquot the filtered supernatant using a syringe into sealable tubes and seal them.

• fill each tube completely with cell medium or add PBS.

5. Ultracentrifuge in a fixed rotor at 10 0000g, 90 minutes, 4 °C.

6. (Optional step) Resuspend the pellet in 1 ml of PBS and ultracentrifuge again at 100 000g, 90 minutes, 4 °C either in smaller tubes or in larger tubes supplemented with PBS.

7. Collect the pellets as described above.

8. To obtain the pure subpopulation of exosomes without any non-exosomal compartments, prepare density gradient for ultracentrifugation using OptiPrep similarly as described above.

QUANTIFICATION OF EVs BASED ON PROTEIN CONCENTRATION

1. Mix 15 µl of exosomes after ultracentrifugation with 15 µl of 20 mM TrisHCl 1% SDS.

2. Vortex.

3. Cool for 30 seconds on ice.

4. Vortex.

5. Break the vesicles with a sonicator – either 3 × 5 min (80 V) with vortexing between sonications or, at a higher power, sonicate for a shorter time.

6. The sample is ready for measuring the amount of proteins using the BCA kit.

PRACTICAL TIPS:

1. When isolating exosomes from cell medium, it is necessary to use EV-free fetal bovine serum (FBS) cell medium. Such a serum is commercially available or can be produced by the above-mentioned procedure.
2. It is also possible to isolate individual subgroups of exosomes using other methods than ultracentrifugation. Commercial kits or separation based on antibodies are used. The choice of the correct isolation procedure should be based on published literature.
3. The collected pellet contains exosomes, which should be stored at -80 °C until the following analyses, e.g., protein quantification, isolation of nucleic acids, transmission electron microscopy and others.
4. When isolating proteins, during sonication, always have ice ready to cool the sample.
5. It is also possible to work with the pellet obtained without purification of vesicles using a density gradient, depending on the subsequent analysis. It is assumed that this pellet contains exosomes with a mixture of non-exosomal particles, which may distort the results of the analyses. For the complete purification of exosomes, it is necessary to perform the mentioned density gradient.

15 Data collection and statistical analysis of data (Ľubica Janovičová, Jakub Szabó)

15.1 Data collection (Ľubica Janovičová)

Data collection is one of the most important steps in the experiment. It can be either exporting data from some measurement in the form of a table or writing down the body weight of experimental animals in a prepared table. Here. it will be shortly described how a data table is prepared and what are the most common issues when working with tables in Microsoft Excel.

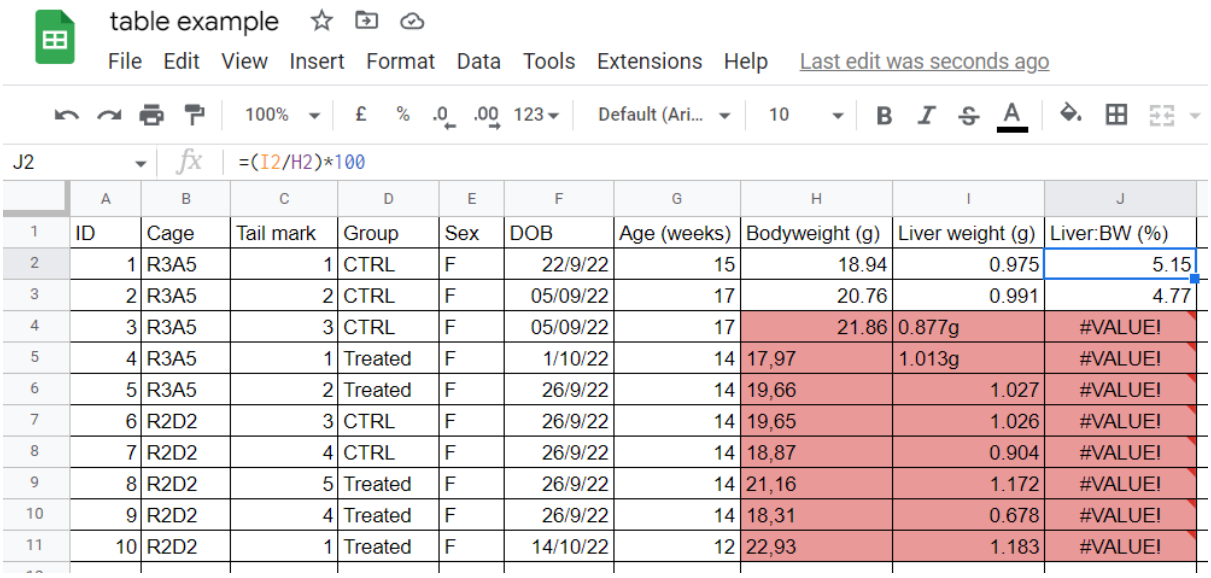
Data table preparation

The data table should be always prepared ahead of the experiment to have it ready when data need to be recorded. Tables in Excel are prepared in such a way that one subject, whether it is a sample of cells, a mouse, or a patient sample, is always in one row. Measured markers and parameters are always going to be found in columns. Please, note in Fig. 56 that one mouse has one row and there are several measurements or information listed in columns.

Many of the descriptive data are crucial such as age, sex, and group assignment. Each mouse has an assigned unique ID which is commonly the first column as this is the most important information to recognize which mouse was which. All samples, e.g., the liver sample are marked with this ID in order to make sure it can be matched to the data. It is not recommended to use cage position or patients name as an ID. The best is always to assign an ID to animals and patients' samples. Here, several common errors in data recording will be listed.

Common data errors

1. Data is in the format of text and cannot be used for calculation (Fig. 56).
2. Dots and commas are used incorrectly for the language setting you use in your sheets. This is problematic for those who use more than one language and those languages that use dots and commas differently.
3. Units are always listed in the first row next to the measured parameter, never next to the number. If units are listed next to the number excel or sheets cannot use it for calculation since it is considered text and not a number (Fig. 56).



	A	B	C	D	E	F	G	H	I	J
1	ID	Cage	Tail mark	Group	Sex	DOB	Age (weeks)	Bodyweight (g)	Liver weight (g)	Liver:BW (%)
2	1	R3A5	1	CTRL	F	22/9/22	15	18.94	0.975	5.15
3	2	R3A5	2	CTRL	F	05/09/22	17	20.76	0.991	4.77
4	3	R3A5	3	CTRL	F	05/09/22	17	21.86	0.877g	#VALUE!
5	4	R3A5	1	Treated	F	1/10/22	14	17,97	1.013g	#VALUE!
6	5	R3A5	2	Treated	F	26/9/22	14	19,66	1.027	#VALUE!
7	6	R2D2	3	CTRL	F	26/9/22	14	19,65	1.026	#VALUE!
8	7	R2D2	4	CTRL	F	26/9/22	14	18,87	0.904	#VALUE!
9	8	R2D2	5	Treated	F	26/9/22	14	21,16	1.172	#VALUE!
10	9	R2D2	4	Treated	F	26/9/22	14	18,31	0.678	#VALUE!
11	10	R2D2	1	Treated	F	14/10/22	12	22,93	1.183	#VALUE!

Fig. 56 Datasheet which shows issues when a comma is used instead of a dot for decimal points. This can be a problem in the opposite order too, a dot is used instead of a comma.

4. In some cases, numbers are changed to date due to the format of the date. This happens for the date format where day, month, and year are separated by a dot (dd.mm.yyyy). In this case, excel or sheets will give incorrect results for the calculation (Fig. 57). When you click on these problematic cells you can see on the function fx row that cell H6 is a date. In Fig. 56. you can see the calculation that uses the date correctly. In this example, the age of mice is calculated by this function: **=(TODAY()-F2)/7**
5. Missing values are not 0. Let's say that we forgot to measure the weight of the liver in our experiment. We leave out the cell empty, but this does not mean that the weight of the liver was 0 g. Despite that, we understand this excel does not and will use it for calculation. In the example shown in Fig. 58 the calculation of liver to body weight ratio is done despite the missing value as it uses zero. These data errors need to be removed. If we flip it and the body weight is missing, the calculation is not possible since that would mean we need to divide by 0.

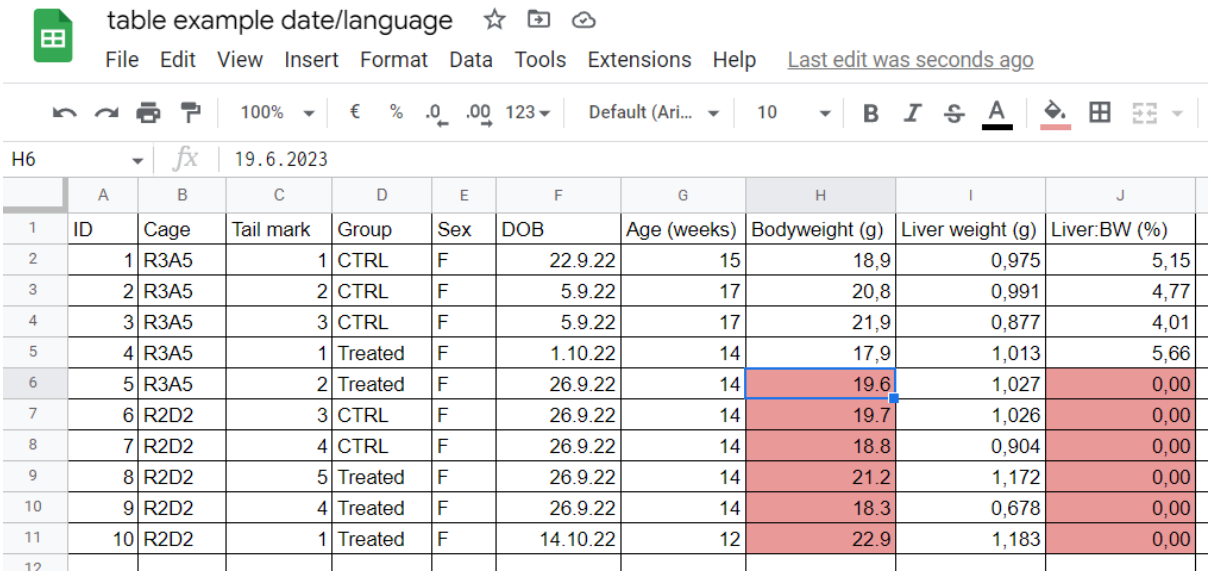


table example date/language

File Edit View Insert Format Data Tools Extensions Help Last edit was seconds ago

H6 | 19.6.2023

	A	B	C	D	E	F	G	H	I	J
1	ID	Cage	Tail mark	Group	Sex	DOB	Age (weeks)	Bodyweight (g)	Liver weight (g)	Liver:BW (%)
2	1	R3A5	1	CTRL	F	22.9.22	15	18,9	0,975	5,15
3	2	R3A5	2	CTRL	F	5.9.22	17	20,8	0,991	4,77
4	3	R3A5	3	CTRL	F	5.9.22	17	21,9	0,877	4,01
5	4	R3A5	1	Treated	F	1.10.22	14	17,9	1,013	5,66
6	5	R3A5	2	Treated	F	26.9.22	14	19.6	1,027	0,00
7	6	R2D2	3	CTRL	F	26.9.22	14	19.7	1,026	0,00
8	7	R2D2	4	CTRL	F	26.9.22	14	18.8	0,904	0,00
9	8	R2D2	5	Treated	F	26.9.22	14	21.2	1,172	0,00
10	9	R2D2	4	Treated	F	26.9.22	14	18.3	0,678	0,00
11	10	R2D2	1	Treated	F	14.10.22	12	22.9	1,183	0,00

Fig. 57 Example table in a format which uses Slovak language and date format dd.mm.yyyy. Here, you can see that DOB is changed to a different format. Mistakes are marked in red where body weight is in date format and therefore the calculation is incorrect.

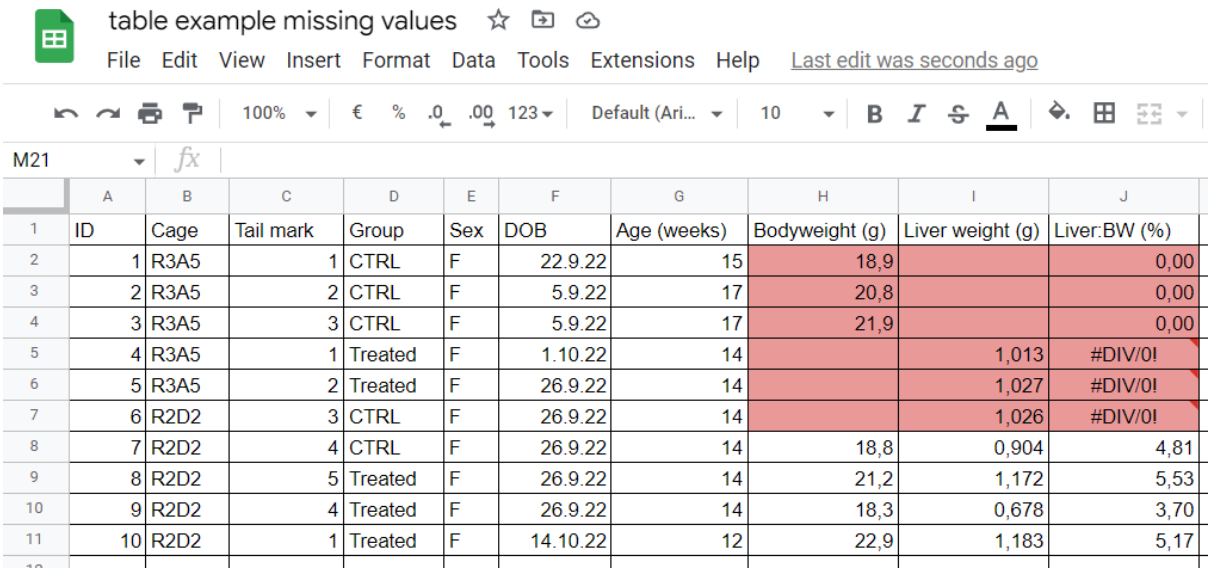


table example missing values

File Edit View Insert Format Data Tools Extensions Help Last edit was seconds ago

M21

	A	B	C	D	E	F	G	H	I	J
1	ID	Cage	Tail mark	Group	Sex	DOB	Age (weeks)	Bodyweight (g)	Liver weight (g)	Liver:BW (%)
2	1	R3A5	1	CTRL	F	22.9.22	15	18,9		0,00
3	2	R3A5	2	CTRL	F	5.9.22	17	20,8		0,00
4	3	R3A5	3	CTRL	F	5.9.22	17	21,9		0,00
5	4	R3A5	1	Treated	F	1.10.22	14		1,013	#DIV/0!
6	5	R3A5	2	Treated	F	26.9.22	14		1,027	#DIV/0!
7	6	R2D2	3	CTRL	F	26.9.22	14		1,026	#DIV/0!
8	7	R2D2	4	CTRL	F	26.9.22	14	18,8	0,904	4,81
9	8	R2D2	5	Treated	F	26.9.22	14	21,2	1,172	5,53
10	9	R2D2	4	Treated	F	26.9.22	14	18,3	0,678	3,70
11	10	R2D2	1	Treated	F	14.10.22	12	22,9	1,183	5,17

Fig. 58 Missing values are not 0. However, excel will use it for calculation in this case since it uses it as 0 being divided by a larger number. If this is flipped, excel gives an error as it cannot divide by 0.

6. Using graphs in excel or sheets for recalculation is needed for most of the measured data such as proteins, ELISA, and many markers. These use the standard calibration and generally, you

can prepare one file that you can use for the recalculation of all proteins. However, you need to understand how the calculation works in order to avoid mistakes. The most common mistake is flipping the x and y axis. Since we use the function (2.89*x-0.031) where we substitute x we should have raw data, in this case. OD measurement on x axis. On the other hand, on y axis are the known standard values highlighted in green (Fig. 59). For correct calculation, it should not be forgotten to subtract the blank measurement from both, sample raw data and standard raw data (highlighted in blue).

	A	B	C	D	E	F
1	ID	OD1	OD2	OD mean	OD minus blank	protein (ug/ul)
2	1	0,766	0,524	0,645	0,529	1,50
3	2	0,985	0,96	0,973	0,857	2,44
4	3	1,073	1,003	1,038	0,922	2,63
5	4	1,033	0,996	1,015	0,899	2,57
6	5	0,593	0,589	0,591	0,475	1,34
7	6	0,571	0,538	0,555	0,439	1,24
8	7	0,487	0,649	0,568	0,452	1,28
9	8	0,386	0,449	0,418	0,302	0,84
10						
11	standards	(ug/uL)	OD1	OD2	OD mean	OD minus blank
12	A	2,00	0,782	0,784	0,783	0,667
13	B	1,50	0,607	0,721	0,664	0,548
14	C	1,00	0,483	0,509	0,496	0,380
15	D	0,75	0,376	0,441	0,408	0,293
16	E	0,50	0,290	0,284	0,287	0,171
17	F	0,25	0,208	0,231	0,219	0,104
18	G	0,13	0,163	0,171	0,167	0,051
19	H	0,03	0,127	0,132	0,130	0,014
20	I	0,00	0,115	0,116	0,116	0,000

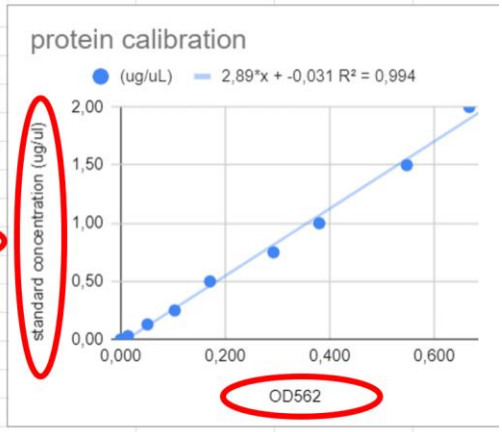
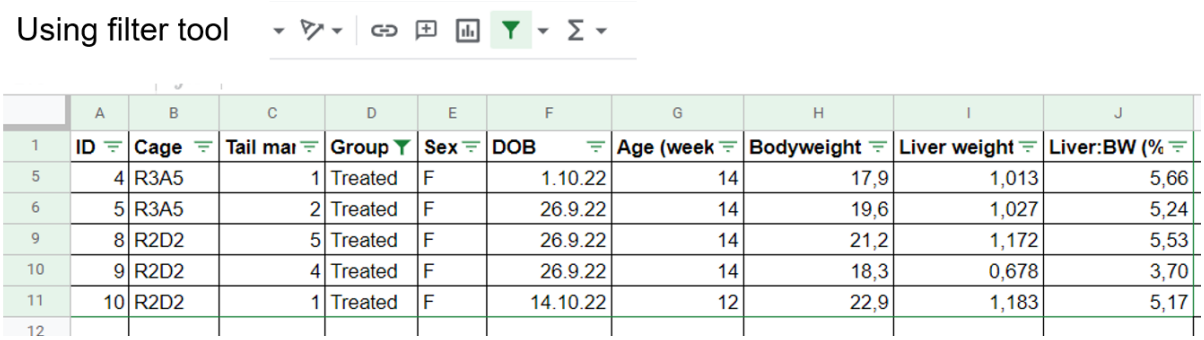


Fig. 59 Protein calculation using standards. Issues that may arise when preparing a graph such as flipped axis and not subtracting blank are circled in red.

Tips for data collection

1. Does the data make sense?
    a. When you look at the recalculated data does it make sense? Sometimes we make mistakes but those can be easy to spot. If the data is off and mice have e.g., 10-fold more of a measured maker is good to think about a possible source of error.
    b. Check for errors in calculation and look for missing values.
2. Filtering the data
    c. If a large data set is analyzed, it is useful to use filter tools in excel or sheets (Fig. 60).

d. Always name the same groups' same way. If you name controls from the first cohort as CTRL and controls from the second cohort as CONTROL those will be filtered separately. The same goes if there are any typos.



	A	B	C	D	E	F	G	H	I	J
1	ID	Cage	Tail mar	Group	Sex	DOB	Age (week	Bodyweight	Liver weight	Liver:BW (%
5	4	R3A5	1	Treated	F	1.10.22	14	17,9	1,013	5,66
6	5	R3A5	2	Treated	F	26.9.22	14	19,6	1,027	5,24
9	8	R2D2	5	Treated	F	26.9.22	14	21,2	1,172	5,53
10	9	R2D2	4	Treated	F	26.9.22	14	18,3	0,678	3,70
11	10	R2D2	1	Treated	F	14.10.22	12	22,9	1,183	5,17
12										

Fig. 60 Filter tool in excel is used to filter based on e.g., groups, sex, age, and all other variables or markers found in the dataset.

3. Using conditional formatting
    a. Conditional formatting is a way to color cells based on the numbers. It means that if we wanted to find cells that are smaller than 0 we could use this tool to find these cells quickly.
4. Do not forget to back up your data! To prevent loss of data it is highly recommended to back it up on google drive, cloud, or an external hard drive.

15.2 Statistical analysis of data (Jakub Szabó)

In general, statistics revolve around collecting, analyzing, and interpreting empirically sampled data. Empirical data are any form of data acquired via observation or experimentation and they represent an essential backbone of a scientific process. However, the world around us, being as complex as it is, makes it difficult to draw any straightforward interpretations of the events we would like to understand. Often misinterpreted as skepticism, modern science understands this complexity and requires conclusions based on solid evidence with special regard to uncertainty and variation in mind. These represent fundamental ideas of statistics and to address them properly, we operate with probability. A lot of time, statistics are misunderstood to provide proof that an event a person observes is true, which is incorrect. Statistics rather provide the probability of observing such an event. Due to

this, scientific data rarely lead to an absolute conclusion and enough independent evidence has to be accumulated supporting the initial hypothesis to accept it as a proper explanation. The statistical approach utilized in basic scientific reasoning uses probability-based methods to evaluate a result and draw a conclusion from it. Traditionally, these calculations were done by pen-and-paper and by using formulas the computations run on, but in today's time, a plethora of statistical software does the computation for the user. Thus, for this manual, we will go through the most widely used software with brief descriptions and we will discuss the most basic approaches to data analysis.

15.2.1 Statistical software

To navigate the wide range of the offered statistical software products, it is essential to divide them based on attributes of interest. The main one is arguably the accessibility of a given software. The usage of most statistical software is monetized, either by a subscription paid regularly or by buying a traditional license for use. However, open-access statistical software or tools are already of very high quality too (often even surpassing paid software), ranging from user-friendly software such as JASP, Jamovi, or SAS to script-operated solutions such as R (and R studio), Shogun, or DataMelt. Despite this, paid statistical software with a strong history of usage remains the most widely used in research. These include software such as GraphPad Prism, IBM SPSS, Stata, Base SAS, Statista, and countless others. Since Graphpad Prism is the most commonly used software at the IMBM, as well as the most widely used software in the field of Biomedical research as it is, we will use it as an example in subsequent practices.

15.2.2 Defining the dataset

Before we can approach any actual analysis, it is essential to take a step back and reflect on what type of data we have on our hands. This is needed mainly to choose the correct method for analysis down the line. Generally, we divide data into **qualitative** and **quantitative**. The difference is that qualitative data cannot be measured numerically, but they are rather sorted by categories, which is why they are often referred to as categorical data. They include audio-visual, symbolic, or text information and can

be further classified into nominal and ordinal data. **Nominal data** lack any order or quantitative properties, such as hair or eye color, marital status, nationality, or gender, and most of the time, they are used to label and sort the data overall. On the other hand, **ordinal data** have a certain ordering, which means they can be defined and described based on a relative value. This positions them somewhere in between qualitative and quantitative data, however, they only show sequences, preventing us from approaching them arithmetically. Mainly, they include data of opinions – feedback, experience, satisfaction, lettered grades, economic status, and educational level. The line between nominal and ordinal variables can be dynamic sometimes, which causes a degree of confusion. For example, we could operate with the educational level (primary – secondary – higher) in a form of a nominal variable, if we only want to categorize the responders for further analysis, but we could also operate with the educational level in a hierarchical order (secondary education being considered "higher" compared to the primary education) and drawing interpretations about our sample of off it, treating it as an ordinal variable.

Quantitative variables are more straightforward in this manner – they are countable and thus, expressed in numbers, making them appropriate for statistical manipulation and visualization in graphs and charts. All quantitative data are continuous, meaning they can take any value within a range. Some sources further separate qualitative data into **continuous** and **discrete**, the main difference being that continuous variables take the form of fractional numbers, while discrete variables take the form of integers or whole numbers. Defining what data and variables we have on our hands is important to understand how to approach the subsequent analysis. However, it also has a more practical reason. A big number of statistical software require data to be clearly defined before being able to work with them and even though some of them already have implemented tools that define data type automatically, it is not rare that they evaluate them incorrectly. Thus, it is important to check before approaching further analysis.

15.3 Descriptive statistics

Deciding what analysis is the most appropriate to use with the data at hand requires knowing what we want to learn from them. Based on our intent, statistical techniques generally fall into either descriptive statistics or inferential statistics. Inferential statistics aim to model events, make judgments, define associations in the dataset and generalize the findings to a larger population based on the smaller sample and we will focus on that later on. Descriptive statistics, as the name suggests,

aim to describe, show and summarize features of the dataset and allow the researcher to understand their data better. To achieve this, we can look at our dataset and its distribution from three points of view:

- Frequency,
- Central tendency,
- Variability.

Frequency, mostly operating with percentages or numbers, summarizes every possible value a variable has in our dataset.

Central tendency operating with mean, mode, and median, estimates the central value of a variable in our dataset. Mean is the most common way of estimating an average value and you get it by adding all recorded observations and dividing them by the number of the observations. The mode tells us what value is the most frequently observed across the recorded values. The median gives us a value that resides in the exact numerical center across the recorded values. For example, if we recorded the height of 5 people (167 cm, 170 cm, 159 cm, 172 cm, 170 cm), the mean would fall on 167.6 cm (838/5), mode on 170 cm (observed twice) and median on 170 cm as well (central value after ranking from lowest to highest).

Variability (often called dispersion as well), operating with range, standard deviation, and variance, gives us the idea of how spread out the values in the dataset are. Range defines how far from each other the extreme values (lowest and highest value) reside and we will get it by subtracting the lowest value from the highest value. Standard deviation explains how far from the mean each separate value lies. To work out the standard deviation, you would need to subtract the mean from each value, square it, average the squared results, and take their square root, which will be equal to the standard deviation of the dataset. Lastly, variance defines the degree of spread of the values in the dataset exhibit and you will get it if you square the standard deviation you just computed. The further the variance is from the mean, the bigger the spread the data exhibit. Naturally, you do not need to calculate these by yourself – the mentioned software does it all for you. In GraphPad Prism, you would choose the Column data table, fill in your values in a respective column, hit the *Analyze* button, choose *Descriptive statistics* and check every option you desire to be produced. An example of this could be observed in Fig. 61.

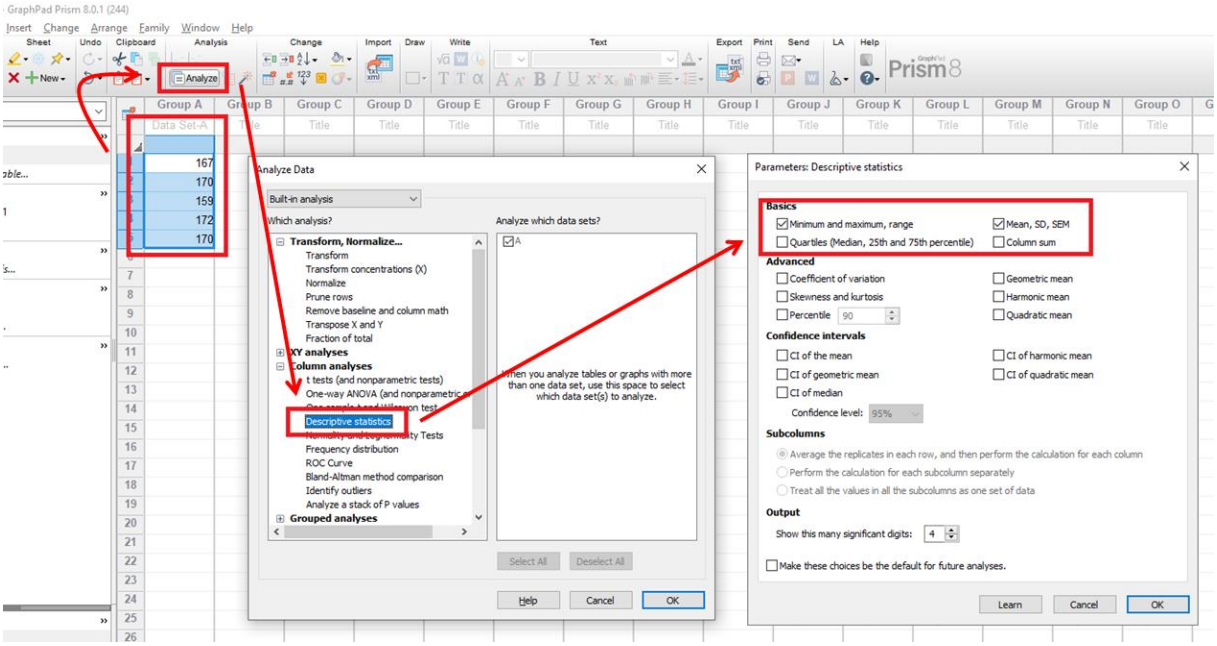


Fig. 61 Computing the Descriptive statistics of the dataset in GraphPad Prism software.

15.4 Distribution and normality

When we put all these descriptive parameters together, we can talk about the distribution of our dataset, or more specifically, the variables we use. Understanding our distribution is imperative when we want to use inferential statistical methods to test hypotheses we have regarding the data. Many naturally occurring variables (height, birth weight, IQ) assume what we call a **normal distribution**, also called Gaussian (named after Karl Gauss, a famous mathematician who described it) or bell-shaped (due to the outline of its curve, when plotted) distribution (Fig. 62). It is specific for the normal distribution that the mean, median, and mode lay on the same value, the distribution exhibits one main peak, and is thus symmetric around the mean – half the values fall below the mean and half above the mean. Since it is symmetric, the proportions of each standard deviation (described by the *z*-score) are consistently equal on both sides of the distribution. Due to normally distributed variables being so common in the world around us, the majority of proper statistical tests are designed for normally distributed variables (also called **parametric tests**). However, there are variables that will not assume a normal distribution, but rather a **non-normal distribution**. Examples of these could be coffee consumption, or bacteria growth, which follows an exponential distribution.

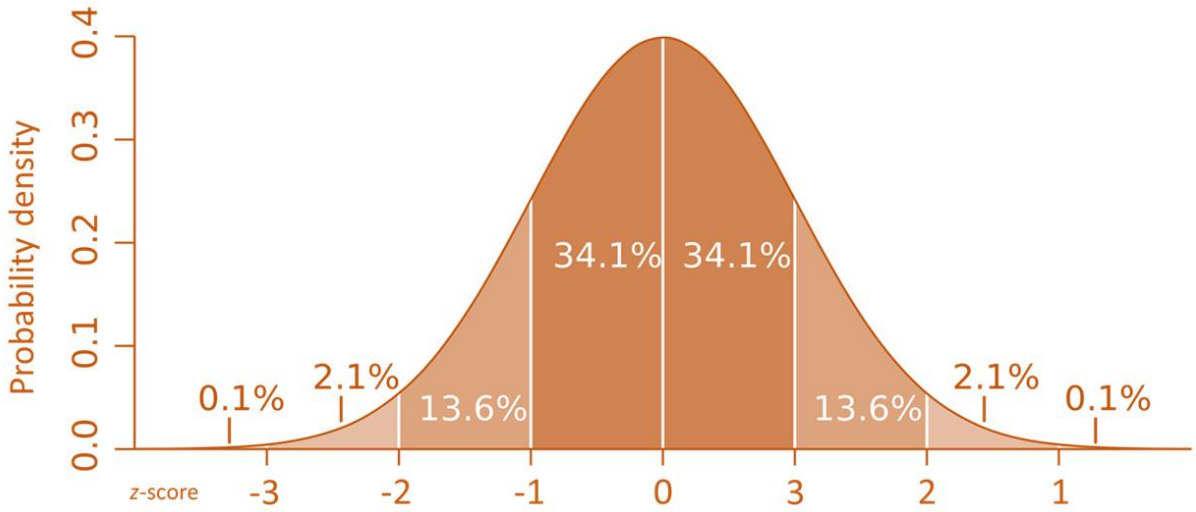


Fig. 62 Gaussian distribution.

Non-normal distribution lacks symmetry and much more often includes extreme values, which skews the distribution. However, there is nothing inherently wrong with it and we have inferential tests that are designed to deal with non-normal distributions (called **nonparametric tests**), but they offer less flexibility, applicability, and precision than the preferred *parametric* tests. Due to this, it is common practice to try and transform data using various **standardization** (normalization) methods to fit a normal distribution. These include evaluating outliers and ruling out possible technical (error in the measurement) or contextual outliers (robustly differs from the rest of the distribution, e.g., due to secondary pathology), logarithmization, or calculating the square root for each data point. In case such transformation does not help data assume the normal distribution, it is to utilize the nonparametric tests to evaluate given hypotheses. The normality of the distribution can be evaluated using specific tests of normality. In GraphPad Prism, choosing a *Column* data table, filling in the values, hitting the Analyze button, and choosing *Normality and Lognormality Tests* allow us to utilize options that evaluate the normality of the data. An example can be observed in Fig. 63. However, there is a catch – tests of normality are very sensitive to sample size and can thus perform inconsistently. It is recommended to not rely solely on them but pair them with a Q-Q plot, a P-P plot, or a histogram to additionally observe if the distribution follows the bell curve. Finally, a tremendously useful paradigm can be also applied in case the sample size of our dataset exceeds a certain threshold called **Central Limit Theorem**. Central Limit Theorem states that means taken from a sufficiently large sample will assume normal distribution even when the population we draw from does not. Simplified, in case our sample exceeds N of 30 (generally, but also partially depending on the context), we can assume normal distribution and use the parametric tests to evaluate our hypotheses.

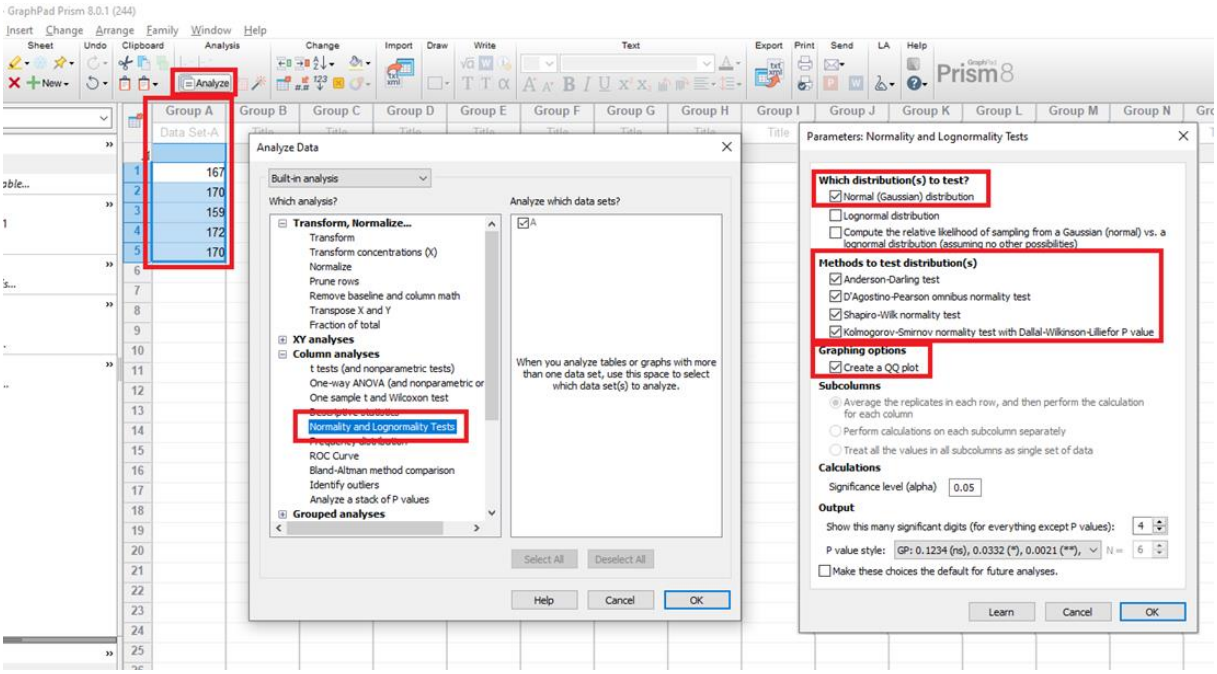


Fig. 63 Tests of normality and plotting a Q-Q plot in GraphPad Prism software.

15.5 Inferential statistics, statistical significance, and practical implications

Following paragraphs will discuss the use of inferential statistics to evaluate our dataset for any hypotheses we might have. As we mentioned earlier, inferential statistics use analytical tools for providing interpretations about a population by examining random samples drawn from it. They aim to model events observed in general or specific (e.g., clinical) populations in a smaller and more practical setting and then try to generalize the findings. We use a plethora of different analytical tools of inferential statistics (tests) based on the nature of the data we work with and based on what we want to learn from our data. The vast majority of these methods are hypothesis-testing, or in other words, tests based on **statistical significance**.

Statistical significance in itself is a topic for a separate work, but for this textbook, we will approach it on a basic level. Every observation and calculation in real life or in a testing environment is burdened with a certain possibility that the event we recorded is caused by an error, or by pure chance, rather than by the effect we are trying to assess. Even in the most apparent situations, we cannot rule out that a factor outside of our control is manipulating the result. Due to this, it is important to estimate the degree to which we can rely on the result not being affected by chance. A measurement that is used for this purpose, widely known as the *p*-value, represents the probability of error having an effect on the observed outcome and is loosely defined as a function of the means and standard deviations in the

dataset. Generally speaking, statistical significance is widely understood as a way of determining that a certain result we observe using methods of inferential statistics is a result that is not burdened by chance or an error. However, this is not so correct; statistical significance refers to a threshold (or rather a set of thresholds) defining a portion of probability (indicated by the *p*-value) of our results being due to a mere chance, which we decide to accept as low enough. The scientific community agreed that less than a 5% probability our results are caused by a chance is sufficient for this purpose, thus the famous expression $\boldsymbol{p < 0.05}$ (the probability of error is lower than 5 %). An interesting fact is that the 5% threshold is not based on any statistical reasoning. It comes from a famous statistician and geneticist Ronald Fisher, who set the value arbitrarily when discussing a different statistical issue. The scientific community adopted it, expanded it to two more thresholds (1 % and 0.1 %), and provided the thresholds with a proper symbol nomenclature widely used in scientific papers across the world today. The said nomenclature goes as follows:

* = $p < 0.05$ (the probability of error is lower than 5%),

** = $p < 0.01$ (the probability of error is lower than 1%),

*** = $p < 0.001$ (the probability of error is lower than 0.1%).

Now that we discussed the main argument in inferential statistics, we can discuss how to utilize it. Listing, describing and explaining all tests largely exceed the scope and intention of this text. Therefore, rather than explaining their theoretical basis and understanding, the following will be a concise protocol describing how to navigate GraphPad Prism to carry out the most basic and commonly used inferential tests and when it is appropriate to use them. It is highly recommended to search for their basic theoretical background individually to have a better grasp on their specific use and the assumptions they require to run effectively.

15.5.1 Student's T-test, Mann-Whitney U-test and Wilcoxon Signed-Rank test

Student's T-test is arguably the most known statistical test. and its understanding is quite common. The T-test is a parametric test; therefore, it is used when the data follow a normal distribution. It calculates the variance of exactly two separate groups (e.g., high-school students and university students), compares them, and decides if the differences between them are statistically significant. It is important to highlight that the T-test can only be used if precisely 2 groups (or rather 2 separate variances) are being compared, no more than that. For this comparison, T-test uses a *t*-value, which can be loosely understood on a dimension – a larger *t*-value means more difference between the groups, smaller *t*-value means more similarities between the groups. It is important to report this value as a part of the statistical notation in the results of any experiment, alongside the *p*-value, reflecting the probability of an error and the *degrees of freedom* (or *df*), reflecting the sample size and factors included (Fig. 64). An example of such notation would be:

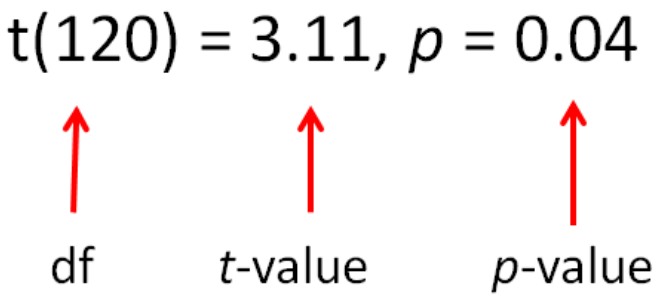


Fig. 64 Statistical notation for Student's T-test.

In the sentence, we could say "*A difference between the red- and blue-shirted lumberjacks was observed in wood-cutting speed [t(120)=3.11. p=0.04]"*. Depending on what we are using the T-test for, we characterize:

- **Independent Samples T-test**, which compares means of two separate groups.
- **Paired Sample T-test**, which compares the mean of the same group under different conditions (e.g., 1 year apart).
- **One Sample T-test**, which compares the mean of our dataset against a known mean (e.g., how different is our group from 0).

Mann-Whitney U-test is a nonparametric version of the Student's T-test; therefore, it is used when the data follow a non-normal distribution. There are more differences between the two tests, but most

importantly, the U-test does not compute any specific value to compare its variance between the two groups, but rather ranks the values of both groups and compares if one group is generally higher-ranked than the other. However, it does compute the *U*-value, which loosely describes the number of times the values of one group precedes the values of the other group in the ranking. Once again, it is important to report it in the statistical notation, which follows the same pattern as for the T-test, just omitting the degrees of freedom (df). To give an example sentence, we could say *"A difference between the red- and blue-shirted lumberjacks was observed in wood-cutting speed (U=100. p=0.04)"*. Finally, the **Wilcoxon Signed-Rank test** is a paired sample form of the U-test, therefore, it is to be used when the same dataset is tested in 2 different conditions (e.g., 1-year apart). To compute a T-test or a U-test in GraphPad Prism, you would choose a *Column* data table, fill in the values, hit the *Analyze* button, and choose *t tests (and nonparametric tests)*, which will provide options for the *Unpaired* and *Paired* experimental design, as well as the option to choose if the data follow a normal distribution (*Yes. Use a parametric test.*) or a non-normal distribution (*No. Use a nonparametric test*). An example can be observed in Fig. 65.

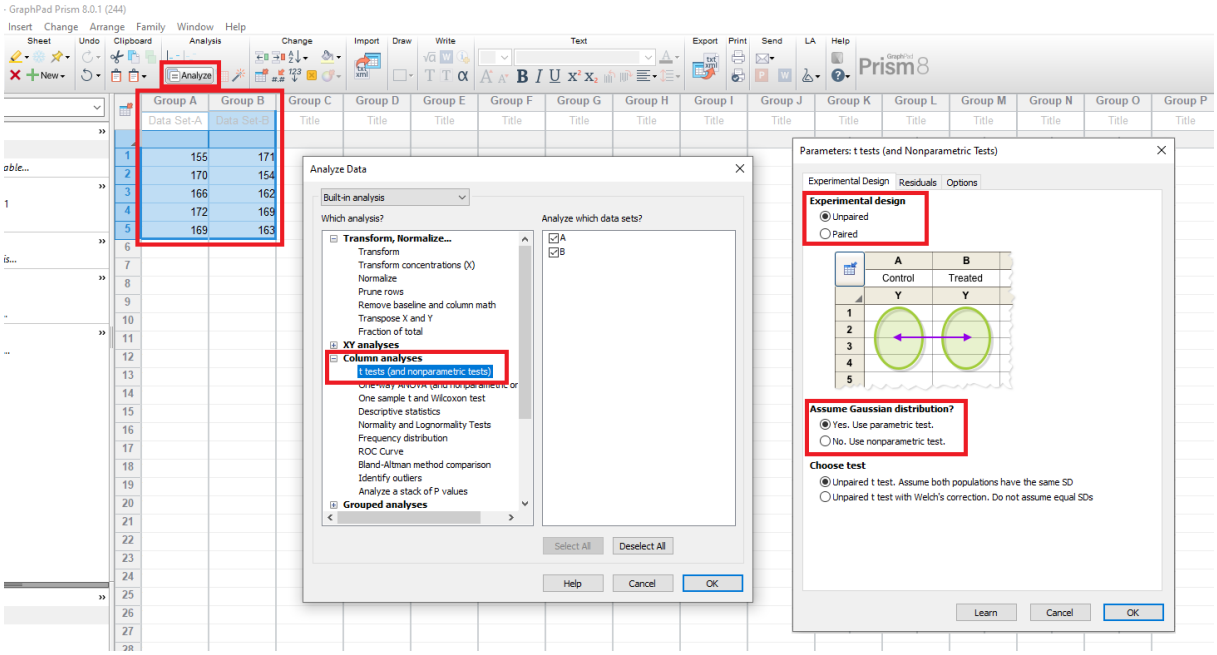


Fig. 65 Computing a Student's T-test and Mann-Whitney U-test in GraphPad Prism software.

15.5.2 One-way ANOVA. Kruskal-Wallis One-way ANOVA and Friedman test

One-way Analysis of Variance (ANOVA) is best described as a more complex version of the Student's T-test, the main difference being that with One-way ANOVA, means of 3 and more groups are compared (you can compare only 2 groups with the T-test) and it is determined if the differences between the groups are statistically significant. It is important to highlight that the One-way ANOVA is an omnibus statistic, meaning it only describes if there is a difference between all of the groups, but will not specify which of the groups are statistically significantly different. After reading through the previous parts of this text, you could come up with an idea to use a series of T-tests to find out which specific groups make up the differences. However, as you surely understand by now, we decide to accept a 5% chance of error as a cut-off point in our analyses ($p < 0.05$). In this manner, each test you conduct will deal with a 5% error rate, and conducting a series of these tests multiply this error. For example, carrying out one T-test means accepting a 5% probability of an error, carrying out two T-tests means accepting a 10% probability of an error, while carrying out three T-tests means accepting a 15% probability of an error (actually, it is not as simple as adding 5% with each test, so with three T-tests, the probability would fall on roughly 14.3%). Such numbers represent an unacceptable probability of an error and One-way ANOVA controls for these errors, so the threshold remains at 5 %, To determine which groups produced the significant difference. One-way ANOVA has built-in **post-hoc tests.** These specify the comparisons between separate groups and tell you exactly how each comparison looks like. Despite the correction, post-hoc tests should be only run if One-way ANOVA returns a significant difference between the groups and when it does, only 1 post-hoc test should be used. There are several post-hoc tests you could choose to use, however, most of the time, you will be using either **Tukey's honestly significant difference (HSD) test** or **Bonferroni Correction.** The differences between them (and all the other post-hoc tests) are more complex than this text intends to be, but for the sake of choosing the more correct one in a specific situation – Tukey's HSD test has more power when testing large numbers of means, whereas Bonferroni has more power when the number of comparisons is smaller (it is also arguably too conservative, often over-correcting the subsequent error probability). The statistical notation is similar to the notation of the T-test, the difference being that instead of *t*-value, an *F*-value is reported, and the degrees of freedom consists of two values, not just one. In the case of One-way ANOVA, it is required to follow up with the results of the post-hoc test, mentioning respective group means, ± standard error of the mean (SEM), and the *p*-value of respective partial comparisons. In a sentence, we could say *"A difference between the red-. blue- and green-shirted lumberjacks were observed in wood-cutting speed [F(2. 118)=78.2, p=0.04). Red-shirted lumberjacks were faster (10.1 ± 1 sec.) compared to both blue- (20 ± 2 sec., p=0.03) and green-shirted lumberjacks (22.5 ± 1.4 sec., p = 0.02). No differences were observed between blue- and green-shirted lumberjacks (p = 0.85).*

Kruskal-Wallis One-way ANOVA is a nonparametric alternative to the parametric One-way ANOVA. Similar to the One-way ANOVA being an extension of the T-Test, Kruskal-Wallis One-way ANOVA is an extension of the Mann-Whitney U-test. Three and more groups are ranked based on their individual values and the analysis checks if the values of one group precede the values of the other groups in the ranking significantly. To correct for the increasing probability of error and specify the differences between individual groups, once again, there are several post-hoc tests that can be used, the most commonly used being the Dunn post-hoc test. The statistical notation is the same as in the case of the parametric One-way ANOVA, only instead of the *F*-value being reported, the χ^2-value is reported, followed by the multiple partial comparisons of respective groups.

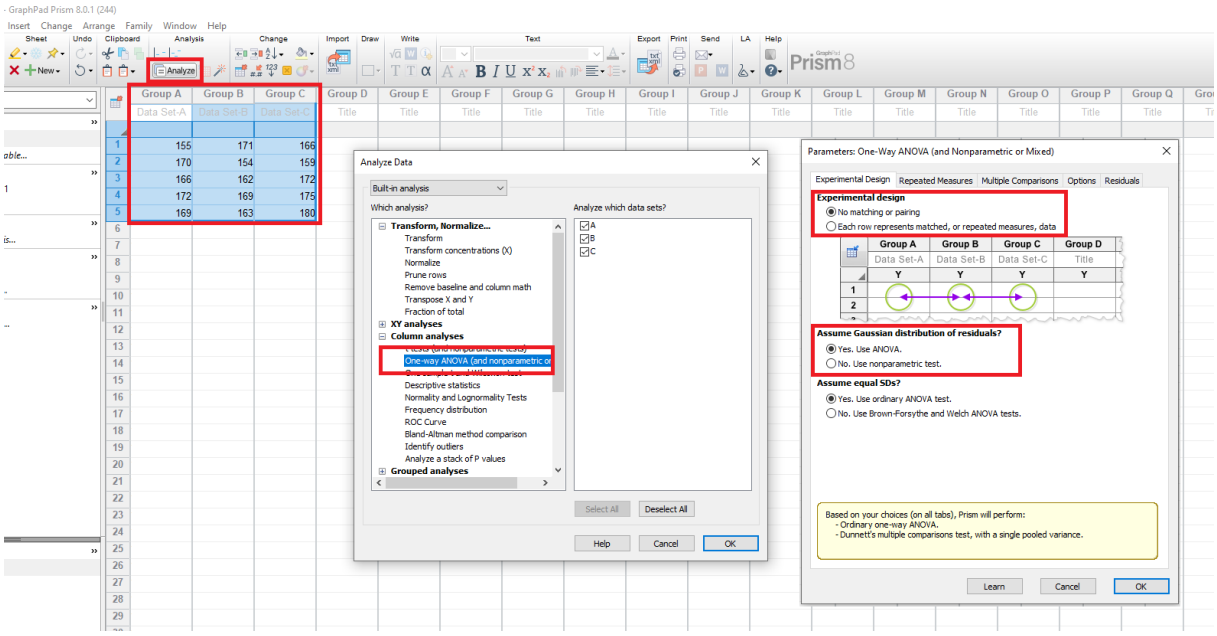


Fig. 66 Computing a One-way ANOVA. Kruskal-Wallis One-way ANOVA and their repeated measures equivalents in GraphPad Prism software.

Using the same approach that was used in the case of the T-tests, group means of the same group recorded in three (and more) different conditions (e.g., baseline, 1-year after, 2-years after) are evaluated using the parametric **Within-subject One-way ANOVA** and its non-parametric equivalent, the **Friedman test**. For Within-subject One-way ANOVA, the *F*-value is utilized for the statistical notation, and for the Friedman test, χ^2-value is used in its place. To compute a One-Way ANOVA in GraphPad Prism, you would choose a *Column* data table, fill in the values, hit the *Analyze* button, and choose *One-way ANOVA (and nonparametric or mixed)*, which will provide options for the between groups (*No matching or pairing*) or within-subject (*Each row represents matched, or repeated measures, data*) alternatives, as well as the parametric (*Yes. Use ANOVA*) or the nonparametric (*No. Use a nonparametric test.*) versions. An example of that could be observed in Fig. 66. More

complicated ANOVA models often combine several additional factors, not just one, as it is in the case of the classical One-way ANOVA. Instead of group differences, these models focus more on the associations between the factors concerning the dependent variable in question. For example, one could choose to look into the differences in time needed to run 100 meters between primary school students, high school students, and university students using a One-way ANOVA, however, they could add in more factors with different levels, such as gender, age, or previous training experience. Depending on the number of factors we include, we refer to the analysis as Two-way (Two-factor) ANOVA, Three-way (Three-factor) ANOVA, and so on. Some of these models even combine between-group factors (such as the level of education) and repeated-measure factors (such as several time points) and these are generally called Mixed ANOVA models.

15.5.3 Correlation

Correlation is at this point largely used even outside statistical or scientific language. In its broader sense, correlation means an association of any kind, however, in statistics, correlation usually refers to a specific degree of the linear relation between two quantitative variables. The fact that the relation between the variables is linear means that change in one variable, whether it is an increase or a decrease, causes the second variable to change for a fixed amount as well. Based on the direction these changes assume we can define three main relations:

- A positive correlation – both variables change in the same direction (Fig. 67 A).
- A negative correlation – an increase in one variable is accompanied by a decrease in the other variable (Fig. 67 B).
- A zero correlation – no statistically significant relationship between the variables can be assumed (Fig. 67 C).

To express the relation between two variables across the whole dataset, we use unit-free measure – correlation coefficients. These are denoted by *r* and range between -1 and 1. Regarding the three main relations we described just now, the *r* value indicates different scenarios:

- Positive *r* values indicate a positive correlation. With one variable increasing, the other increases as well.
- Negative *r* values indicate a negative correlation. With one variable increasing, the other is decreasing.

- The closer *r* is to zero, the weaker the relationship is. In case *r* = 0, there is no relation between the variables in question.

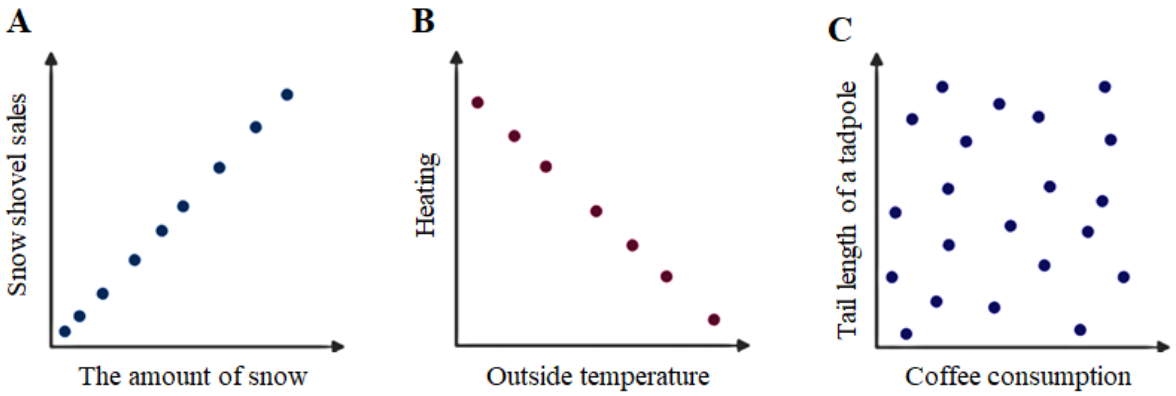


Fig. 67 Three main types of correlations with real-life examples. A. Positive correlation. B. Negative correlation. C. Zero correlation.

In case *r* = 1 or *r* = -1, we observe a causative relationship. In a scientific and statistical sense, it is close to impossible to record a causative relationship in naturally occurring events. Even interventional studies with a rigid and straightforward methodology cannot clearly state that causality has been observed in the experiment. In almost all cases, to establish causality, we need to rely on several repetitions of the same experimental setting and probabilistic reasoning. This is, in part, caused by science being aware of the fact that in such a complex setting the world around us, no factors are truly linear in relation and correlation does not equal causation, no matter how strong it is. Once again, depending on the normality of distribution our dataset assumes, the r takes the form of either the **Pearson correlation coefficient** (*r*, parametric, normal distribution) or the **Spearman's rank correlation coefficient** (*rs*, nonparametric, non-normal distribution). Once again, these are accompanied by the p-value, to indicate what probability of error the result could be burdened with. Finally, since the r coefficient is not the most intuitive way of defining the effect size, we use the **coefficient of determination (R2).** R2 is a squared value of the r coefficient and can be loosely defined as the proportion of variation in the dependent variable that is explained by the effect of the independent variable. To give an example, if R2 = 0.32, we can understand that if the first variable changes by 1, the other changes by 0.32. or in other words, when the second variable changes, 32% of its change can be explained solely by the effect of the first variable. The r coefficient, p-value, and coefficient of determination (R2) make up the statistical notation when reporting the results of the correlation. In the text, we could say "*A strong positive correlation was observed between the physical attractivity and the thickness of the beard in men (r = 0.58. p = 0.01. R2 = 0.336)*". To compute correlations in GraphPad Prism, you would choose either *XY, Column, or Multiple Variables* data table (all work if you want to do basic correlations), fill in the values, hit the *Analyze* button, choose

Correlation under the *XY analyses*, then check to *Compute r for X vs. every Y data set*, deciding what coefficient to use based on the data distribution, parametric correlation (*Yes. Compute Pearson correlation coefficients.*), or nonparametric correlation (*No. Compute nonparametric Spearman correlation.*) and finally, always using the Two-tailed option. An example could be observed in Fig. 68.

After reading through this text, you should have a solid understanding of the basic statistical principles needed when collecting, analyzing, and interpreting data. The text also offers a practical approach to each analysis, which could be followed step-by-step. A decision tree including all the basic inferential tests that can be used can be observed in Fig. 69.

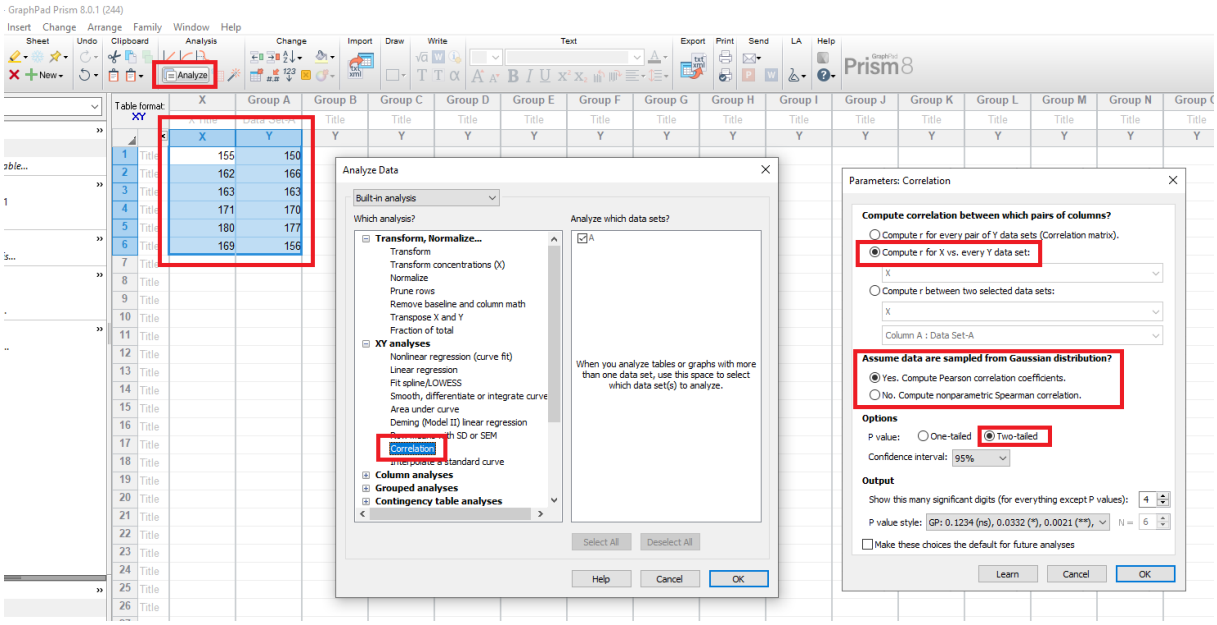


Fig. 68 Computing a correlation in GraphPad Prism.

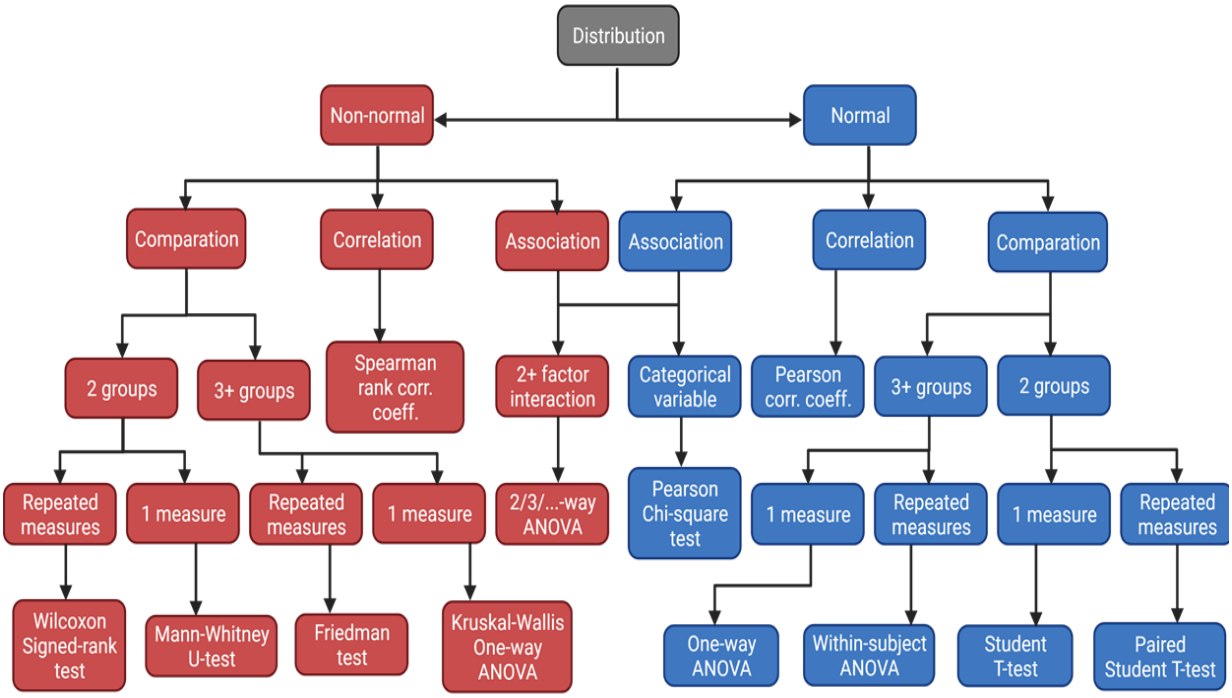


Fig. 69 Deciding what inferential analysis to use.

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