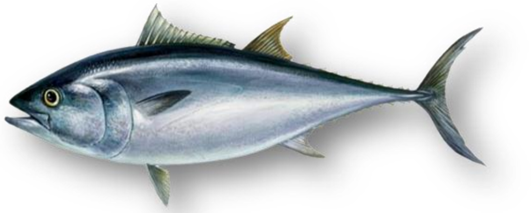
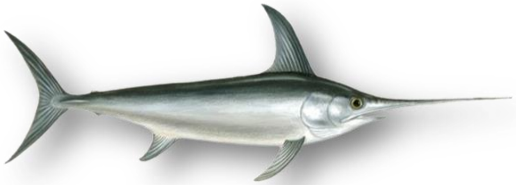
INSTITUT ZA OCEANOGRAFIJU I RIBARSTVO SPLIT

PROTOCOL

Thunnus thynnus and *Xiphias gladius* stomach sampling and DNA quality check





Split, May 2024.

Co-funded by the European Union

Program ZA RIBARSTVO I AKVAKULTURU

MetaGenom

Contents

Executive Summary

This protocol was developed within a scientific study titled “Stomach sampling and analysis for large pelagic species combining morphological and metagenomic approach - METAGENOM” (WP4 of Service contract No 112/2022/VV "Assessing the Impact of Fishing on Marine Habitats and Monitoring Catches in Recreational and Sport Fishing at Sea"). METAGENOM Study is co-financed by the Ministry of Agriculture of the Republic of Croatia and the European Maritime, Fisheries and Aquaculture Fund (EMFAF).

The METAGENOM study focuses on the analysis of stomach contents of large pelagic fish species, specifically the Atlantic bluefin tuna (*Thunnus thynnus*) and swordfish (*Xiphias gladius*), using morphological and metagenomic approaches, and it is divided into three tasks:

- Data collection, sample processing, development of protocols for fish stomach sampling, and quality control checklists for DNA
- Stomach content analysis and establishing the protocol for metabarcoding analysis of fish stomach content
- Analysis of research results and final reporting

This document is Deliverable D4.1, Protocol for *Thunnus thynnus* and *Xiphias gladius* stomach sampling and DNA quality check. It contains descriptions of fish sampling, stomach content sampling for morphological and molecular diet characterization, DNA extraction from stomach contents after visual inspection, and DNA quality control. It presents all the important steps toward obtaining extracted stomach content DNA for further application of DNA metabarcoding analysis (Deliverable D4.2).

Atlantic bluefin tuna and swordfish are important target species for commercial and recreational fisheries. Stomach content analysis provides insight into fish diet and feeding habits, forming the basis for understanding trophic interactions in aquatic food webs and integrating many important ecological components.

Since morphological identification of prey has limitations due to prey digestion, with species composed of soft tissues often being missed, combining this approach with DNA metabarcoding, which delivers a qualitative prey inventory, enables more accurate taxonomic characterization of fish stomach contents. This dual approach overcomes the constraints of traditional methods, providing a comprehensive and detailed picture of the dietary habits of these pelagic species.

Recommended format for purposes of citation:

Žuvić, L. and Šegvić-Bubić, T. 2024. Protocol - *Thunnus thynnus* and *Xiphias gladius* stomach sampling and DNA quality check**.** Deliverable D4.1 in Assessing the Impact of Fishing on Marine Habitats and Monitoring Catches, Service contract No 112/2022/VV, MPS and EMFAF, 31 pgs.

Equipment required for the implementation of the protocol

Key Equipment:

- Knife for field sampling
- Scissors for field sampling
- Tweezers for field sampling
- Digital balance for field sampling
- Tape measure
- Portable cooler or a portable freezer
- -20 ℃ freezer
- -80 ℃ freezer
- 4°C refrigerator
- Autoclave
- UV lamps
- Laboratory tweezers
- Laboratory scissors
- Laboratory vessels (50 ml, 100 ml, 150 ml, 250 ml, 600 ml, 1000 ml)
- Laboratory tables
- Laboratory trays
- Digital camera
- Camera holder
- A ruler
- Ichthyometer
- Histological needles
- Ice machine
- Digital laboratory balance
- Styrofoam box
- Laboratory rack
- Set of laboratory pipettes (0,2-2 µl, 1-10 µl, 2-20 µl, 10-100 µl, 20-200 µl, 200-1000 µl)
- PCR cabinet
- Hand blender
- Laboratory spoon
- Thermomixer/Thermoshaker Biosan TS-100c
- Biosan Vortex V-1 plus
- Eppendorf Centrifuge 5430 R
- Boeco Mini Centrifuge M-6
- Qubit 4 Fluorometer
- Implen NanoPhotometer N50
- Laboratory wipe
- USB flash drive

Key Consumables:

Consumable	Supplier	Quantity/Note	Cat. No
96 % alcohol	BIOVIT d.o.o	2,5l	32294-2.5l
Laboratory gloves (L)	Novodent	100 per package	1193-D
Cuvettes with cap 5ml	INEL	1000 per package	60558
Cuvettes with cap 2ml	INEL	100 per package	72.694.006
Safe lock Eppendorf tubes (1.5 ml)	INEL	1000 per package	0030120.094
Pipette tips volume: 01-20µl	Kemolab	960 per package	F171203
Pipette tips volume: 20-200µl	Kemolab	960 per package	F171503
Pipette tips volume: 200-1000µl	Kemolab	960 per package	F171703
50-milliliter Falcon tubes	INEL	25 per package	62.548.004
Qiagen, DNeasy Blood & Tissue Kit	INEL	for 250 samples	69506
Qubit assay tubes (0.5 mL)	Biosistemi	500 per package	Q32856
Qubit dsDNA BR Assay Kit	Biosistemi	for 100 assays	Q32850

1. Fish sampling on board/landing site

Note - all steps of the protocol are identical for Atlantic bluefin tuna *Thunnus thynnus* (BFT) and swordfish *Xiphias gladius* (SWO), except for the sampling of the first spine of the first dorsal fin for BFT and the second spine of the anal fin for SWO and the sampling of juveniles in the case of BFT.

1.1. After the fisherman has taken the fish (BFT/SWO) out of the sea onto the boat (Figure 1, 2), the abdominal cavity of the caught animal must be opened and the stomach separated from the other organs (Figure 3).

1.2. If possible, determine the sex of the caught individual.

Note - weigh the gonads and store a piece of the gonad in a cuvette with formalin for histological processing.

1.3. Sample the first spine of the first dorsal fin for BFT (Figure 4) or the second spine of the anal fin for SWO (Figure 5) and store it in an envelope with the following data written on it: date of sampling, length of the individual, weight of the individual, registration number of the boat that caught the fish, fishing area where the fish was caught, sex, weight of the gonads and place of landing.

1.4. Tie up the separated stomach (Figure 6) so that the contents do not leak out and store it in a portable cooler with ice or in a portable freezer. If juvenile BFT are sampled, store the whole individual (10 – 40 cm).

1.5. Store the samples in the freezer after arrival at the Institute.

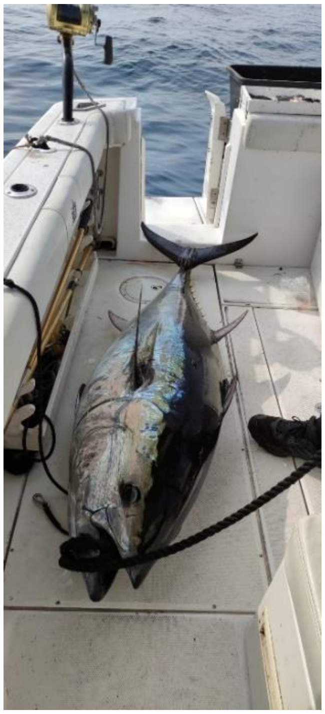


Figure 1. Caught adult Atlantic bluefin tuna (Author: L. Žuvić).

Figure 2. Caught swordfish (Author: L. Žuvić).

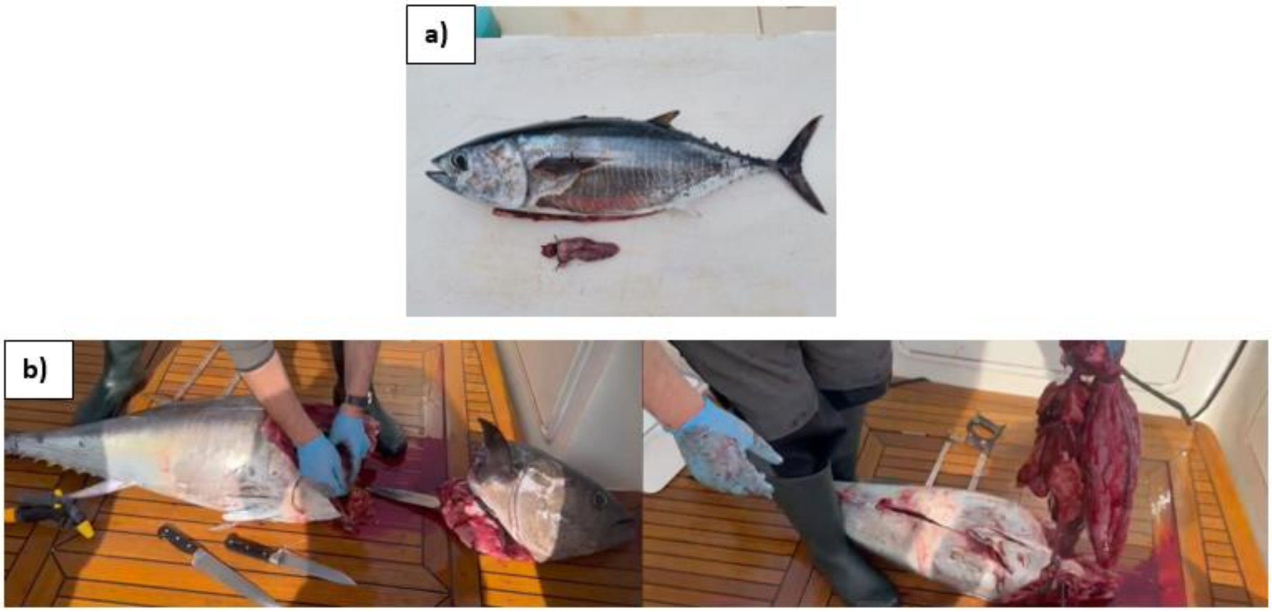


Figure 3. Sampling of the stomach of juvenile (a) and adult (b) BFT individuals (Author: L. Grubišić).

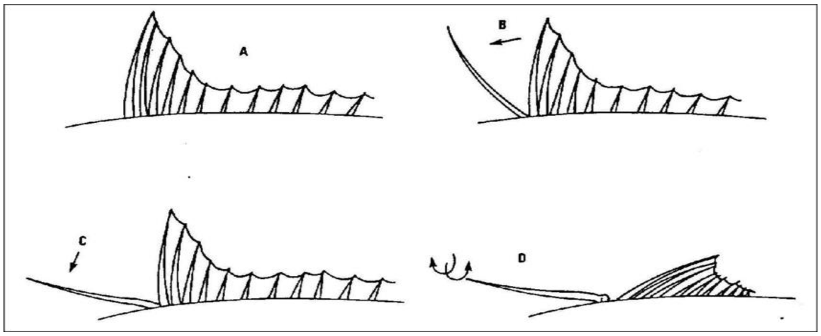


Figure 4. Sampling technique of the first spine of the first dorsal fin of BFT (taken from Compeán - Jiménez and Bard, 1980).

Figure 5. Sampling of the second spine of the anal fin of SWO (Author: L. Žuvić).

Figure 6. Tied up stomach of Atlantic bluefin tuna (Author: L. Žuvić).

2. Morphological sampling of the stomach contents

2.1. All instruments (tweezers, scissors) and glass vessels should be autoclaved and/or treated with UV radiation (UV lamps) before sampling begins. In addition, all surfaces (laboratory tables, trays) should be cleaned with 70% alcohol and irradiated with UV radiation.

2.2. The stomach must be thawed slightly so that the prey in the stomach does not thaw completely (the thawing time depends on the time of year: 4 to 6 hours).

2.3. The digital camera for the photographic documentation of the morphological sampling needs to be fixed on a holder at an angle of 90° (Figure 7).

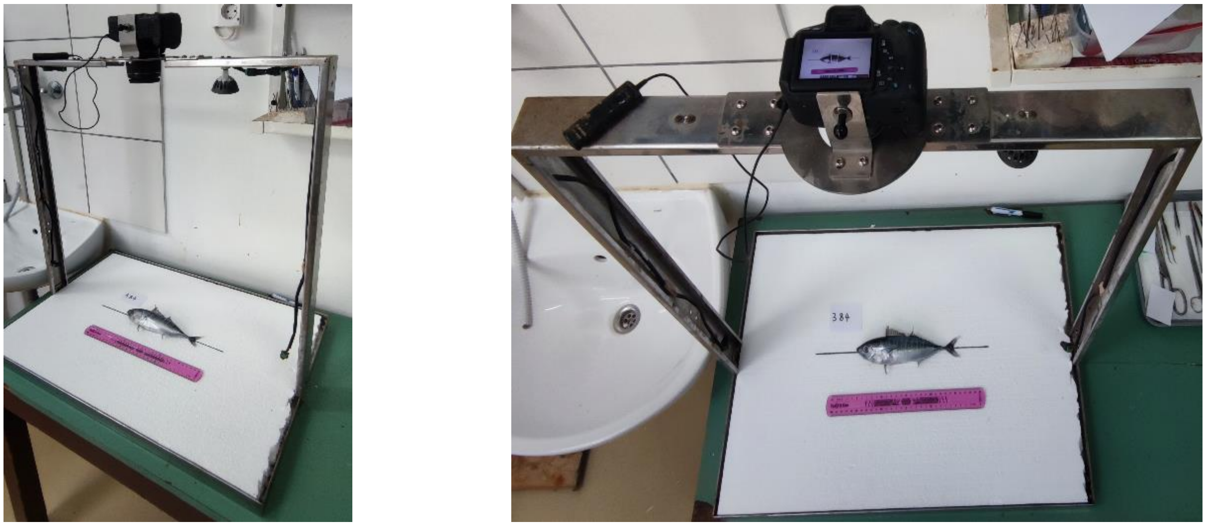


Figure 7. View of the camera holder during sampling of juvenile tuna (Author: L. Žuvić).

2.4. The sampled stomach must be photographed before opening and after opening (with prey) (Figure 8). When photographing, a label with the following information should be attached next to the stomach: sample number, processing date, fishing gear used to catch the individual, registration number of the boat that caught the fish and fishing area where the fish was caught.

Figure 8. Digital photos of the sampled stomach before and after opening (Author: L. Žuvić).

2.5. Measure the length and height of the stomach with an ichthyometer or ruler (Figure 9a), and weigh the mass of the full stomach on a digital laboratory balance (Figure 9b).

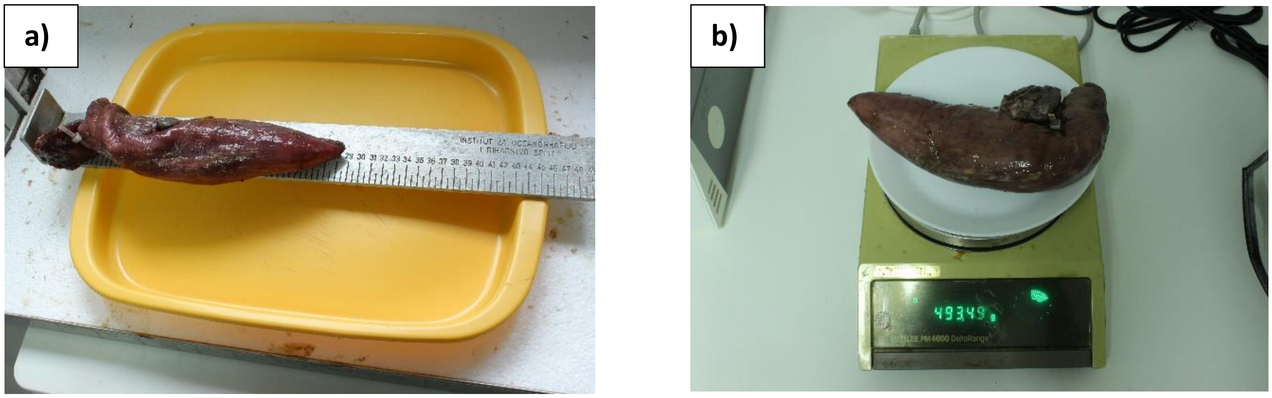


Figure 9. Measurement of the length of the sampled stomach (a) and weighing on a digital laboratory balance (b) (Author: L. Žuvić).

2.6. Based on the filling of the stomach with prey, it is necessary to determine the "stomach fullness", where 0 indicates that the stomach is empty, 1/2 indicates that it is partially full, and 1 indicates that it is completely full.

2.7. Depending on the species, the stomach contents are separated into sterile laboratory vessels (example: we separate all sardines from the stomach contents into the same vessel) (Figure 10), after which the mass of the empty stomach is weighed on a digital laboratory balance.

Figure 10. Separated stomach contents in a sterile laboratory vessel (Author: L. Žuvić).

2.8. Separated stomach contents in sterile laboratory vessels should be identified morphologically to the lowest possible taxonomic category. The contents should be weighed individually by species and the entire contents together (Figure 11).

Figure 11. Weighing the stomach contents on a digital laboratory balance (Author: L. Žuvić).

2.9. After weighing, the separated stomach contents in laboratory vessels put in a styrofoam box with ice (Figure 12) to prevent DNA degradation. The box must first be irradiated with UV radiation (UV lamp) and cleaned with 70% alcohol.

Figure 12. Laboratory vessels with stomach contents in a styrofoam box with ice (Author: L. Žuvić).

Note - when sampling juveniles, in addition to the above, it is necessary to measure the length of the individual with an ichthyometer (Figure 13), weigh the individual on a digital laboratory balance (Figure 14a), photograph the individual in a neutral position with the fins spread (Figure 14b), sample the first spine of the first dorsal fin, * otoliths and store a piece of muscle tissue in a cuvette with 96 % alcohol.

Figure 13. Length measurement of juvenile Atlantic bluefin tuna (Author: L. Žuvić).

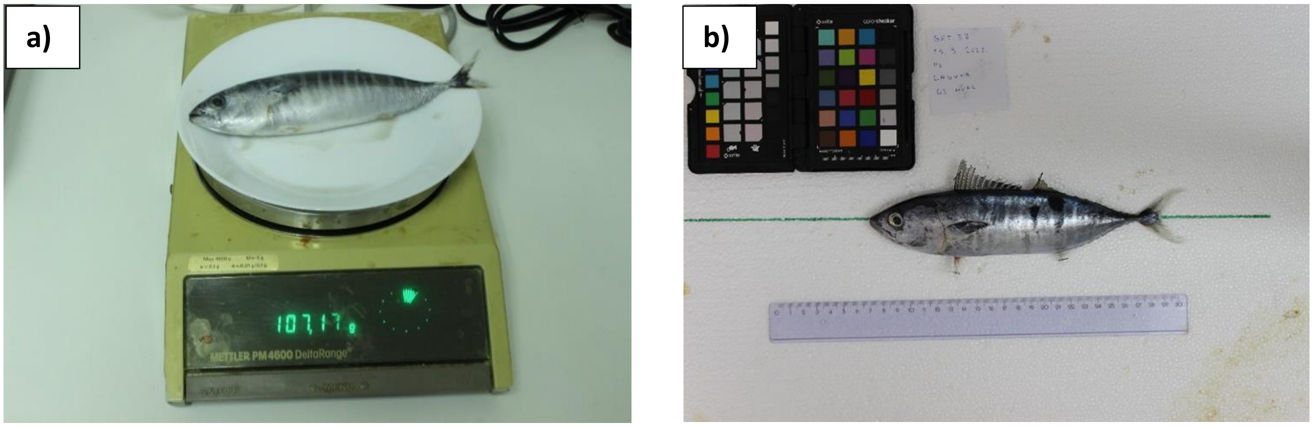


Figure 14. Weighing of juvenile Atlantic bluefin tuna on a digital laboratory balance (a), juvenile Atlantic bluefin tuna in neutral position with spread fins (b) (Author: L. Žuvić).

3. Calculation of dietary indices

For each prey (species of the main taxa preyed upon by the species studied), the following dietary indices are proposed to measure feeding intensity and assess the trophic range of each species:

3.1. Frequency of occurrence (%O), percentage of stomachs with a particular type of prey in relation to the total number of stomachs containing food.

3.2. Numerical (%N) and weight (%W) composition, expressed as percentage of individual prey in total content, in number and weight, respectively.

3.3. Index of relative importance (IRI), IRI = %O (%N+%W), standardized according to %IRI = (IRI/ΣIRI) x100 (Cortes, 1997).

3.4. Index of relative importance prey-specific (%PSIRI = % Fpi (%Npi+ %WPi) 0·5) (Brown et al., 2011).

3.5. Vacuity index (v), calculated as the percentage of empty stomachs divided by the sum of full and empty stomachs, or its opposite, repletion index (R), calculated as the percentage of full stomachs divided by the sum of full and empty stomachs.

3.6. Gastro-somatic index (Ga.SI), Ga.SI=100(total weight of stomach contents/total weight of fish) (Desai, 1970).

3.7. Species diversity in terms of both the number of prey (H'_n) and the prey volume (H'_v), calculated using the classic Shannon-Wiener index:

$$H = -\sum_{i=1}^{S} p_i \log_2 p_i$$

Where *S* is the number of species, p_i is the proportion of species *i* (in number for H'_n and in volume for H'_v) in relation to the total abundance/volume of individuals (i.e. relative abundance or volume of the species). Prey with an abundance of less than 3 % and unidentifiable remains should be excluded from these indices.

4. Molecular sampling of the stomach contents

Before starting the molecular processing of the samples, the work surface and equipment must be prepared (Figure 15), cleaned with 70% alcohol and irradiated with UV lamps. Prepare a rack with safe lock Eppendorf tubes (1.5 ml) (4 tubes per sampled stomach content), which are required for DNA extraction and on which you must write the name and serial number of the processed sample. Equipment that allows autoclaving should be autoclaved.

Figure 15. Laboratory work surface with equipment (Author: L. Žuvić).

4.1. DNA extraction

4.1.1. Each step of the DNA extraction must be performed in the PCR cabinet (Figure 16), with the exception of those steps where this is not possible for technical reasons (step: 4.1.2.).
Note - the PCR cabinet has its own UV lamp for sterilizing the equipment and surfaces inside the cabinet.

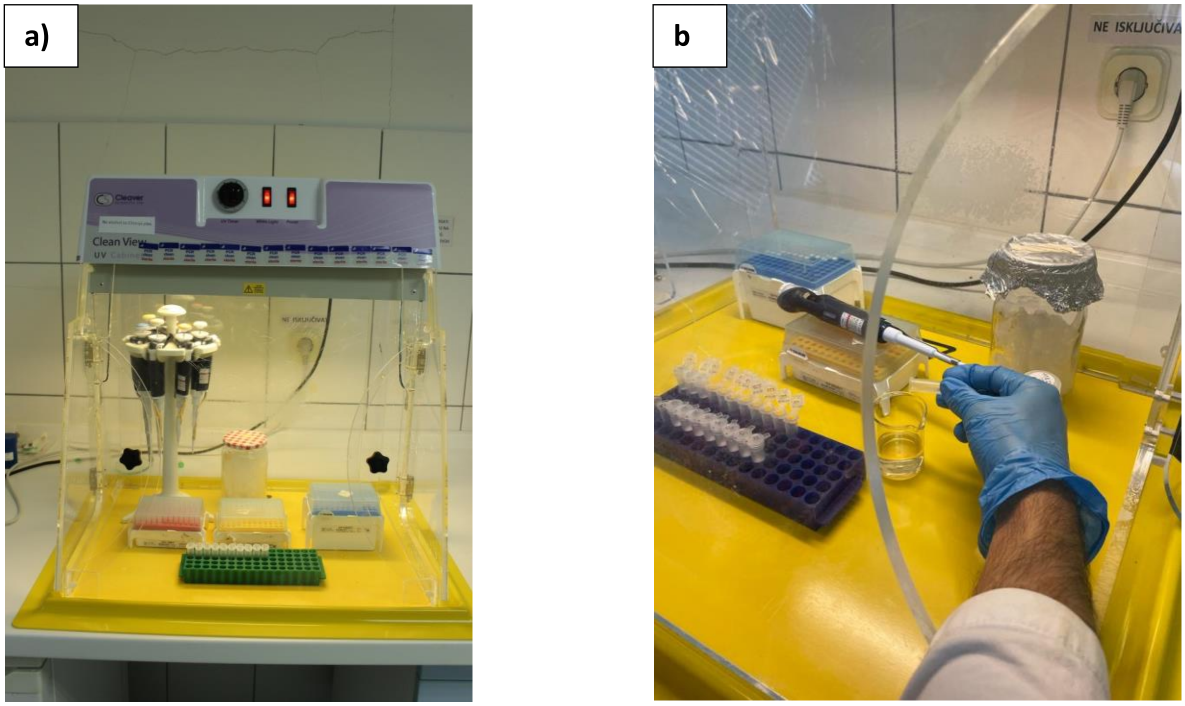


Figure 16. PCR cabinet with laboratory equipment (a), DNA extraction procedure inside the PCR cabinet (b) (Author: L. Žuvić).

4.1.2. Prey separated in laboratory vessels homogenize with a hand blender (Figure 17a) for 30 seconds to 2 minutes, depending on the amount and type of prey. Homogenization is performed in sterile laboratory vessels in a styrofoam box with ice (Figure 17b) to reduce the possibility of DNA degradation during homogenization.

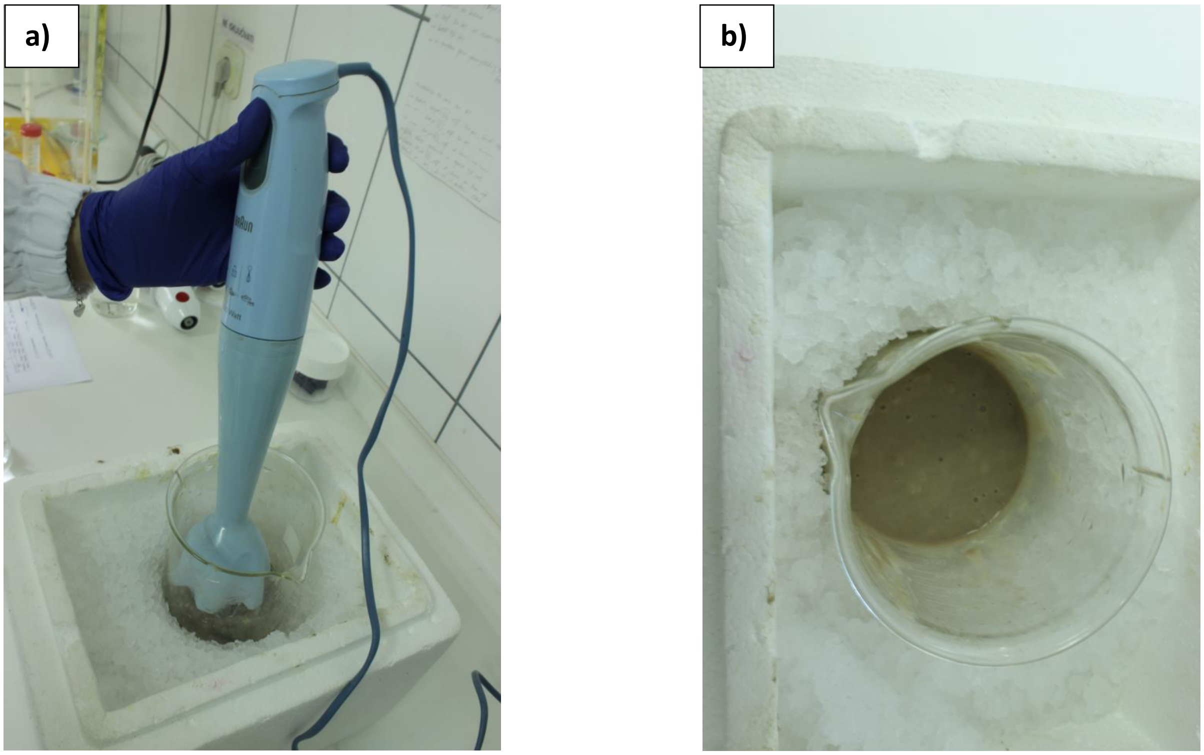


Figure 17. Procedure of homogenization of stomach contents (a), and homogenized stomach contents (b) (Author: L. Žuvić).

4.1.3. Add 180 µl ATL buffer to previously prepared safe lock Eppendorf tubes (1.5 ml). Using a sterile laboratory spoon or a sterile pipette tip, add ~ 150 - 250 mg of the homogenized stomach contents to the tubes (Figure 18). Add 20 µL of Proteinase K (4 mg/mL) or 4 µL of Proteinase K (20 mg/mL). Transfer the remaining homogenate of stomach contents from a sterile laboratory container into 50-milliliter Falcon tubes (Figure 19) and store in a freezer at a temperature of -80 °C.

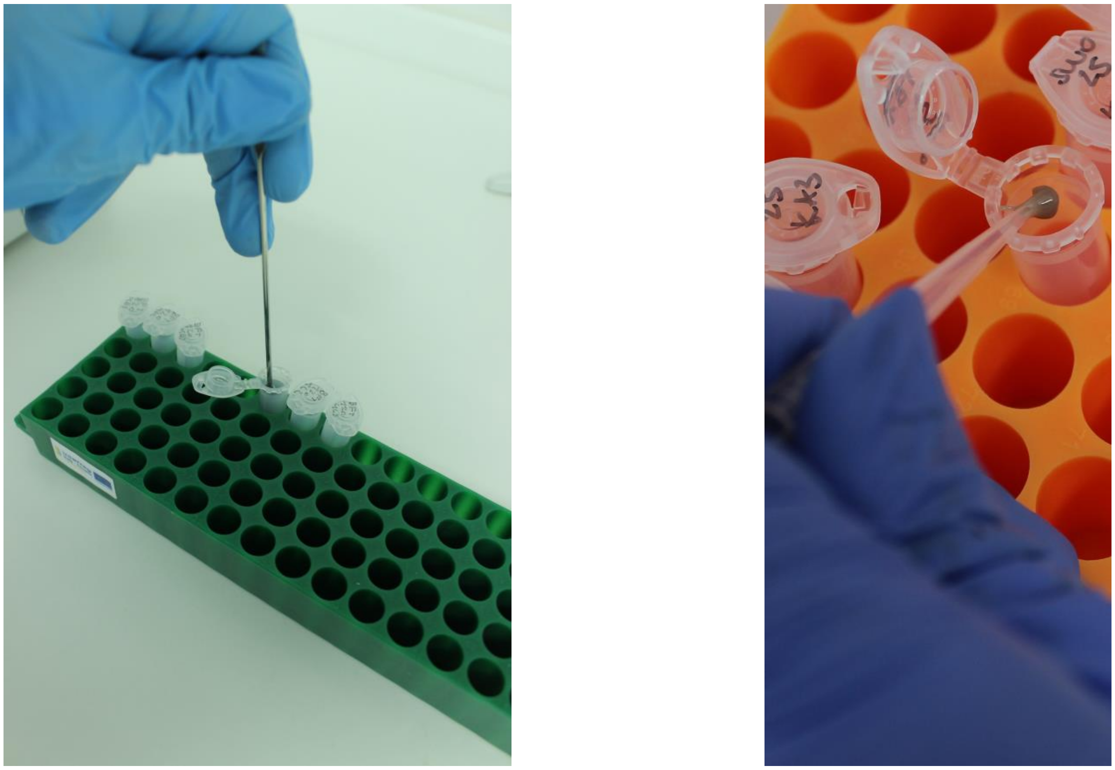


Figure 18. Addition of homogenate of stomach contents in safe lock Eppendorf tubes (Author: L. Žuvić).

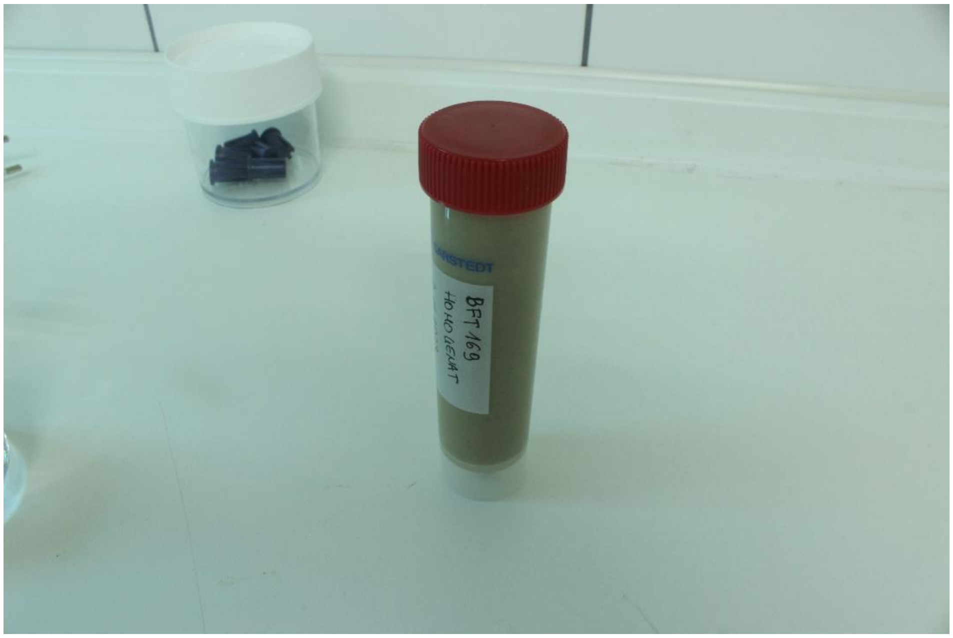


Figure 19. Homogenate of the stomach contents in a 50-milliliter Falcon tube (Author: L. Žuvić).

4.1.4. Place Safe lock Eppendorf tubes in a thermomixer overnight or for at least 4 hours to digest the tissue (temperature: 55-56 °C, number of revolutions: ~ 650 rpm or ~ 800 rpm in the case of a 4-hour digestion) (Figure 20).

Figure 20. Thermomixer with the samples placed for digestion (Author: L. Žuvić).

Note - perform the remaining steps of the DNA extraction according to the manufacturer's (Qiagen) instructions using the DNA extraction kit (DNeasy Blood & Tissue Kit) (Figure 21), with modifications in steps 4.1.6., 4.1.7., 4.1.9.

Figure 21. Qiagen, DNeasy Blood & Tissue Kit (Taken from: https://www.qiagen.com/us/products/discovery-and-translational-research/dna-rna-purification/dna-purification/genomic-dna/dneasy-blood-and-tissue-kit).

4.1.5. After digestion, add 200 µl of AL buffer to the tubes. Mix briefly by vortexing (Figure 22a).

4.1.6. Add 200 µl of ethanol (96-100%). Mix briefly by vortexing, then centrifuge briefly (a few seconds) (Figure 22b) to precipitate impurities.

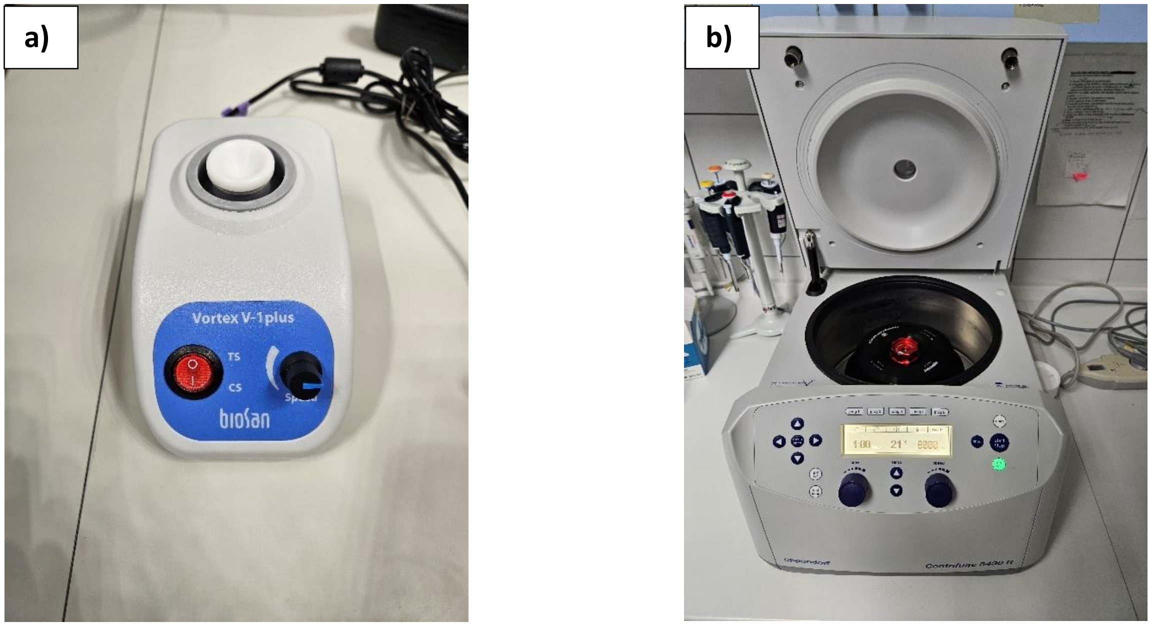


Figure 22. Biosan Vortex V-1 plus (a), Eppendorf Centrifuge 5430 R (b) (Author: L. Žuvić).

4.1.7. Pipette 600 µl of the mixed solution from the tube (make sure to avoid precipitated impurities during pipetting) and transfer it to the DNeasy mini spin column, which is placed in the collection tube (2 ml). Centrifuge for 1 minute (8000 rpm). After centrifugation, discard the collection tube.

4.1.8. Place the DNeasy mini spin column in a new collection tube (2 ml). Add 500 µl of AW1 buffer to the column. Centrifuge for 1 minute (8000 rpm). After centrifugation, discard the collection tube.

4.1.9. Place the DNeasy mini spin column in a new collection tube (2 ml). Add 500 µl of AW2 buffer to the column. Centrifuge for 3 minutes (14000 rpm). After centrifugation, pour the solution out of the collection tube and repeat the centrifugation (14000 rpm) for 3 minutes. After centrifugation, discard the "collection tube".

4.1.10. Transfer the DNeasy mini spin column into a new safe lock Eppendorf tube (1.5 or 2.0 ml).

4.1.11. Elute the DNA by adding 30 µl (2 x 15) AE buffer to the central membrane of the DNeasy mini spin column. Incubate for 1 minute at room temperature (15-20 °C). Centrifuge for 1 minute (8000 rpm). Discard the DNeasy mini spin column and store a safe lock Eppendorf tube with eluted DNA in the freezer at a temperature of -20 °C.

5. Checking the concentration and purity of the DNA

After DNA extraction, the concentration (Qubit fluorometer) and purity (Implen NanoPhotometer) of the DNA must be checked. Before each of the above procedures, the work surface and equipment must be prepared, cleaned with 70 % alcohol and irradiated with UV radiation (UV lamps).

5.1. Concentration measurement with a Qubit fluorometer - preparation of working solutions and samples

5.1.1. Frozen DNA samples must be placed in a refrigerator at 4°C in order to thaw and temper them. When all 4 tubes of DNA extracted from one stomach contents are thawed, combine them in a new safe lock Eppendorf tube, then divide the combined DNA into two safe lock Eppendorf tubes, so that each DNA sample of stomach contents has replicas.

5.1.2. On the rack, prepare one safe lock Eppendorf tube (1.5 mL) for the preparation of the Qubit buffer mix, two Qubit assay tubes (0.5 mL) (Figure 23) for the preparation of the Qubit standard, and Qubit assay tubes depending on the number of samples whose concentration is to be measured.

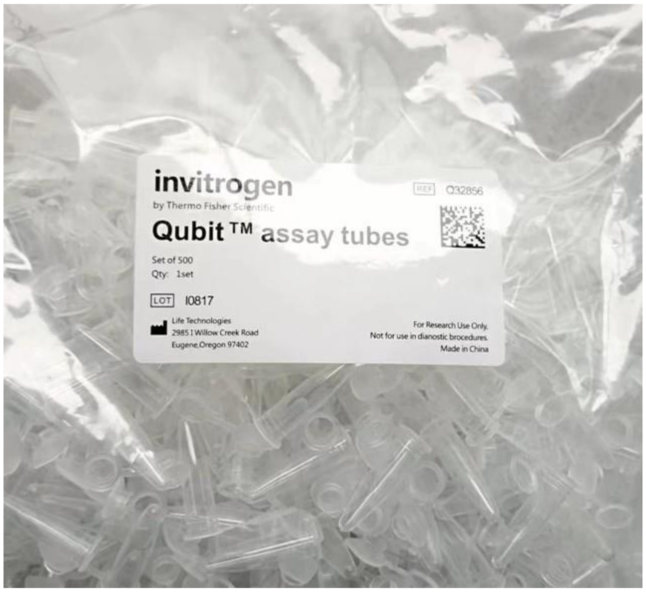
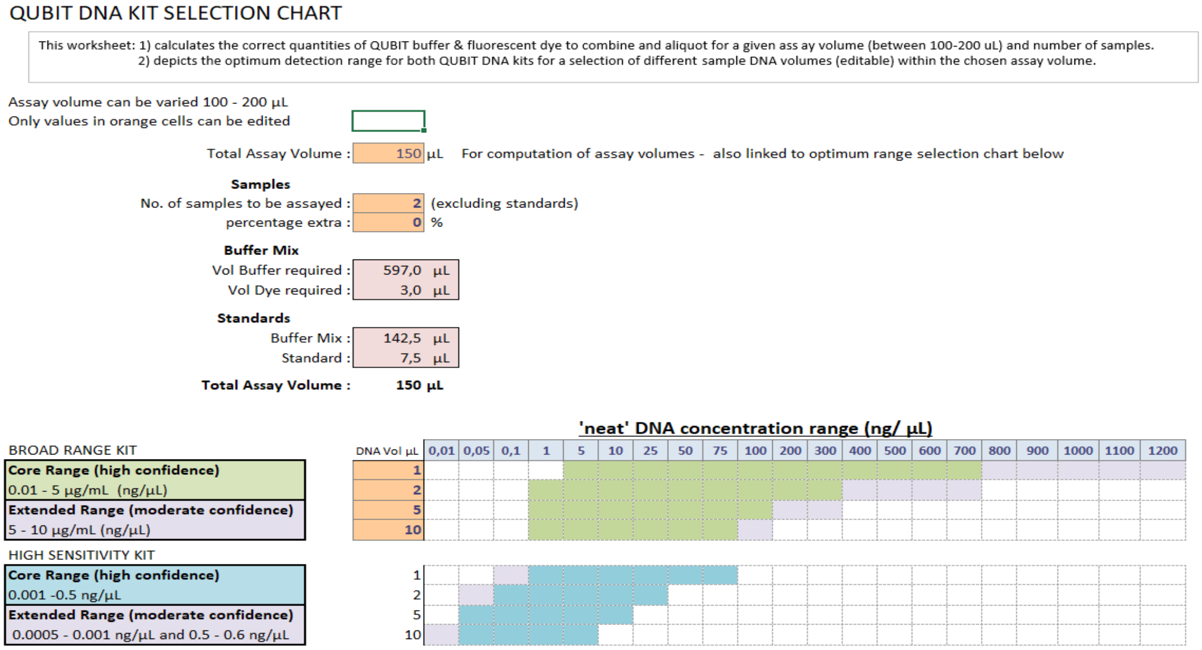


Figure 23. Qubit assay tubes (Author: L. Žuvić).

5.1.3. Using a Microsoft Excel spreadsheet (Figure 24), calculate the volume required to prepare the Qubit buffer mix and the Qubit standards.

5.1.4. In the Microsoft Excel spreadsheet, in the "No. of samples to be assayed" cell, enter the number of samples with which the concentration will be measured (example: 2) by pressing the Enter key in the cells "Vol. Buffer required" (example: 597.0 µl) and "Vol. Dye required" (example: 3.0 µl) are obtained the volumes required to prepare the Qubit buffer mix, while in the "Buffer mix" (example: 142.5 µl) and "Standard" (example: 7.5 µl) cells the volumes required to prepare the Qubit standard are obtained (Figure 24).



QUBIT DNA KIT SELECTION CHART

This worksheet: 1) calculates the correct quantities of QUBIT buffer & fluorescent dye to combine and aliquot for a given ass ay volume (between 100-200 uL) and number of samples.
2) depicts the optimum detection range for both QUBIT DNA kits for a selection of different sample DNA volumes (editable) within the chosen assay volume.

Assay volume can be varied 100 - 200 µL
Only values in orange cells can be edited

Total Assay Volume : 150 µL For computation of assay volumes - also linked to optimum range selection chart below

Samples
No. of samples to be assayed : 2 (excluding standards)
percentage extra : 0 %

Buffer Mix
Vol Buffer required : 597,0 µL
Vol Dye required : 3,0 µL

Standards
Buffer Mix : 142,5 µL
Standard : 7,5 µL

Total Assay Volume : 150 µL

'neat' DNA concentration range (ng/ µL)

BROAD RANGE KIT
🟩 **Core Range (high confidence)** 0.01 - 5 µg/mL (ng/µL)
🟪 **Extended Range (moderate confidence)** 5 - 10 µg/mL (ng/µL)

DNA Vol µL	0,01	0,05	0,1	1	5	10	25	50	75	100	200	300	400	500	600	700	800	900	1000	1100	1200
1					🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟪	🟪	🟪	🟪	🟪
2				🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟪	🟪	🟪	🟪					
5				🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟪	🟪									
10				🟩	🟩	🟩	🟩	🟩	🟩	🟪											

HIGH SENSITIVITY KIT
🟦 **Core Range (high confidence)** 0.001 -0.5 ng/µL
🟪 **Extended Range (moderate confidence)** 0.0005 - 0.001 ng/µL and 0.5 - 0.6 ng/µL

DNA Vol µL	0,01	0,05	0,1	1	5	10	25	50	75	100	200	300	400	500	600	700	800	900	1000	1100	1200
1			🟪	🟦	🟦	🟦	🟦	🟦	🟦												
2		🟪	🟦	🟦	🟦	🟦	🟦														
5		🟦	🟦	🟦	🟦	🟦															
10	🟪	🟦	🟦	🟦	🟦																

Figure 24. Microsoft Excel spreadsheet with the calculated volumes required to prepare the buffer mix and the standards required to measure the concentration with the Qubit fluorometer (Author: J. B. Taggart)

5.1.5. Based on the previously calculated volumes, using the Qubit dsDNA BR Assay Kit (Figure 25), prepare the Qubit buffer mix and Qubit standards.

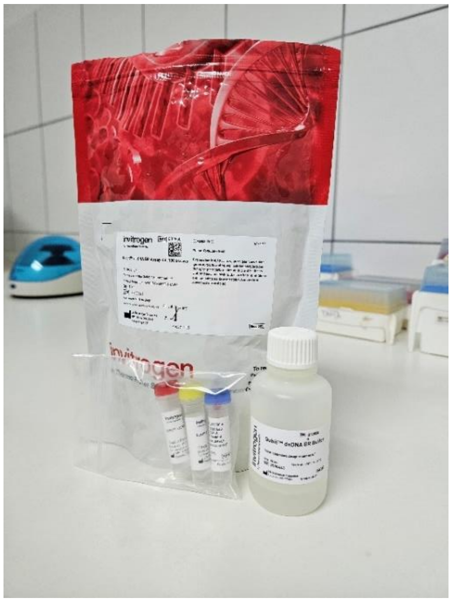


Figure 25. Qubit dsDNA BR Assay Kit (Author: L. Žuvić).

5.1.6. Write the names of the working solutions (example: standard 1, standard 2, buffer mix) and the samples with a marker on the lid of the prepared safe lock Eppendorf tube and the Qubit assay tubes.

Note - Do not label the side of the tubes as this may interfere with the sample reading rule.

5.1.7. Pipette the calculated volume of the Qubit dsDNA BR Buffer (example: 597.0 µl) and the Qubit dsDNA BR Reagent (example: 3.0 µl) into the safe lock Eppendorf tube intended for the preparation of the buffer mix.

5.1.8. Slowly swirl the safe lock Eppendorf tube with the buffer mix up and down so that the solution does not foam. Centrifuge briefly (a few seconds) with a mini centrifuge (Figure 26).

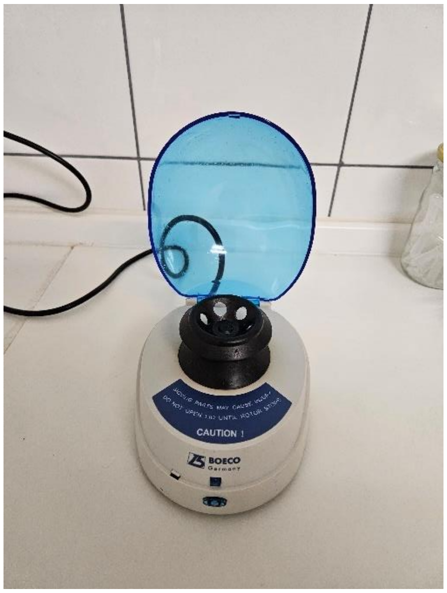


Figure 26. Boeco Mini centrifuge M-6 (Author: L. Žuvić).

5.1.9. Pipette the calculated volume of the buffer mix (example: 142.5 µl) from the safe lock Eppendorf tube containing the prepared buffer mix into the two Qubit assay tubes intended for the preparation of the Qubit standard.

5.1.10. Add the calculated volume of Qubit dsDNA BR Standard # 1 (example: 7.5 µl) to the Qubit assay tube previously labeled on the lid as the tube for preparation of Standard 1 and the same calculated volume (7.5 µl) of Qubit dsDNA BR Standard # 2 to the Qubit assay tube previously labeled as the tube for preparation of Standard 2.

5.1.11. Slowly swirl the Qubit assay tubes containing the prepared Qubit standards up and down so that the solution does not foam. Centrifuge for a few seconds using a mini centrifuge.

5.1.12. Dispense the remaining volume of buffer mix from the safe lock Eppendorf tube into Qubit assay tubes intended for checking the concentration of DNA samples. Pipette 148 µl of the buffer mix from the safe lock Eppendorf tube containing the buffer mix and 2 µl of the DNA from the safe lock Eppendorf tube containing the DNA whose concentration is to be measured into each of the Qubit assay tubes intended for checking the concentration.

5.1.13. Slowly swirl the Qubit assay tubes containing the buffer mix and DNA up and down to prevent the solution from foaming. Centrifuge for a few seconds using a mini centrifuge.

5.2. Concentration measurement with a Qubit fluorometer - reading standards and DNA samples

5.2.1. On the home screen of the Qubit Fluorometer, press dsDNA (Figure 27a) and then select dsDNA Broad Range as the analysis type (Figure 27b).

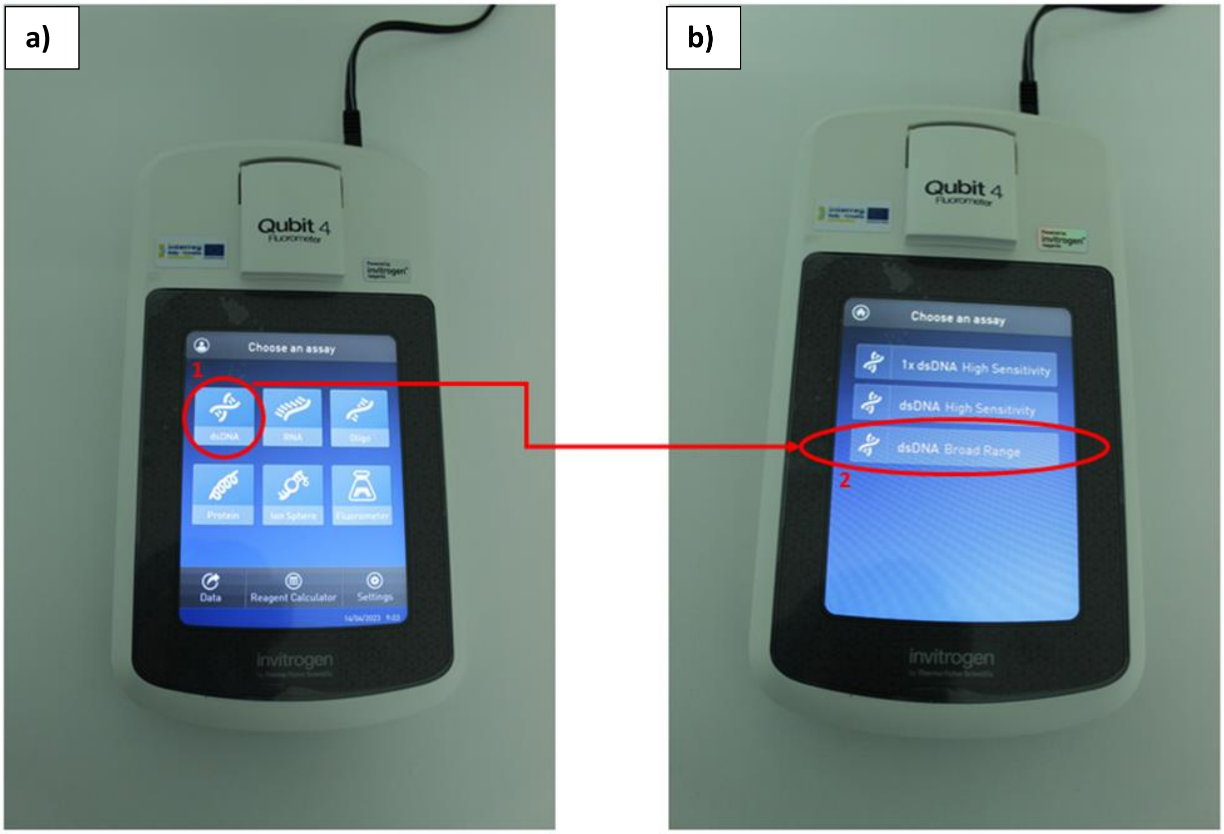


Figure 27. Qubit fluorometer: Home screen (a), screen with analysis options (b) (Author: L. Žuvić).

5.2.2. After selecting the type of analysis, the device displays the screen for reading the standard (Figure 28a). Open the sample chamber and insert the Qubit assay tube containing the standard 1 (Figure 28b), close the chamber (Figure 28c) and then press Read standard (Figure 28a.). When the reading is complete (~3 seconds), remove the standard 1 Qubit assay tube and repeat the process with the standard 2 Qubit assay tube. When the calibration is complete, the device will display a screen with the calibration values (Figure 29).

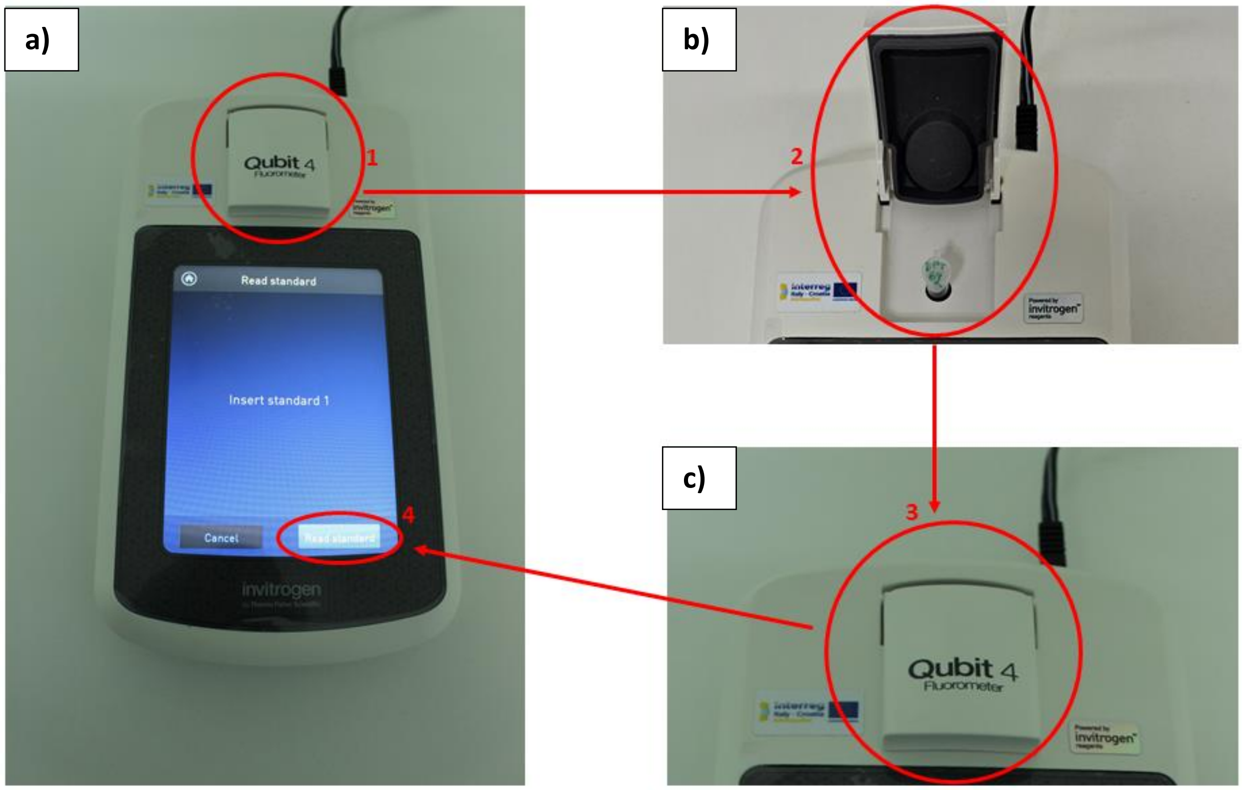


Figure 28. Qubit fluorometer: standard reading screen (a), open sample chamber with Qubit assay tube (b), closed sample chamber (c) (Author: L. Žuvić).

Figure 29. Qubit fluorometer: screen with calibration values (Author: L. Žuvić).

5.2.3. On the calibration values screen, press Run samples (Figure 30a). On the sample analysis screen that appears, select the volume of DNA sample (2 µl) (Figure 30b) that was previously added to the Qubit assay tube with the buffer mix. Select the unit of measurement (ng/µl) in which the read DNA concentration is to be displayed (Figure 30b). Open the sample chamber and insert the Qubit assay tube containing the DNA and buffer mix (Figure 30c), close the chamber (Figure 30d) and then press the Read tube (Figure 31b). When the reading is complete (~3 seconds), remove the Qubit assay tube and the result of the DNA concentration measurement will be displayed on the device screen (Figure 31).

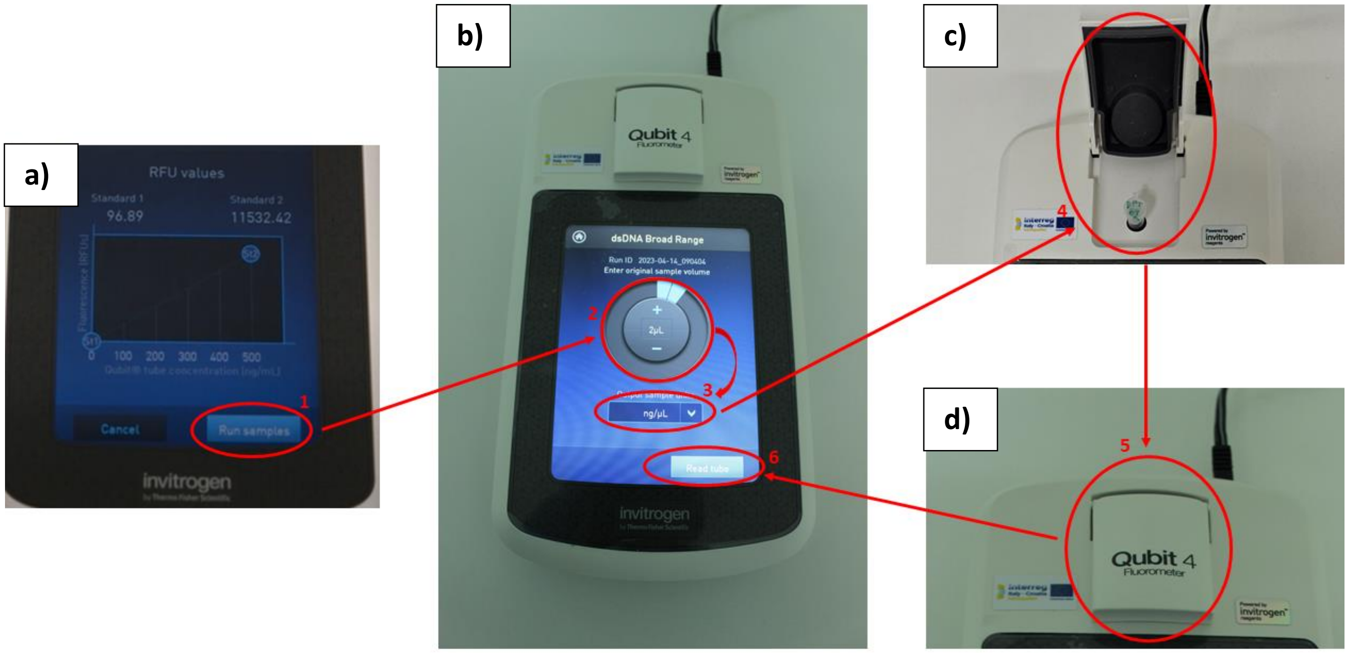


Figure 30. Qubit fluorometer: calibration value screen (a), sample analysis screen (b), open sample chamber with Qubit assay tube (c), closed sample chamber (d) (Author: L. Žuvić).

Figure 31. Qubit fluorometer: screen showing the result of the DNA concentration reading (Author: L. Žuvić).

5.2.4. Repeat the reading procedure with the remaining Qubit assay tubes containing the DNA whose concentration is to be checked.

5.3. Concentration measurement with a Qubit fluorometer - data export

5.3.1. Press Data on the home screen of the Qubit fluorometer (Figure 32a). On the data export screen that appears, press the empty fields to select the data to be exported (Figure 32b). Once you have selected the data to be exported, press Export (Figure 32c). Select the destination for the data export: Cloud, USB (Figure 32d).

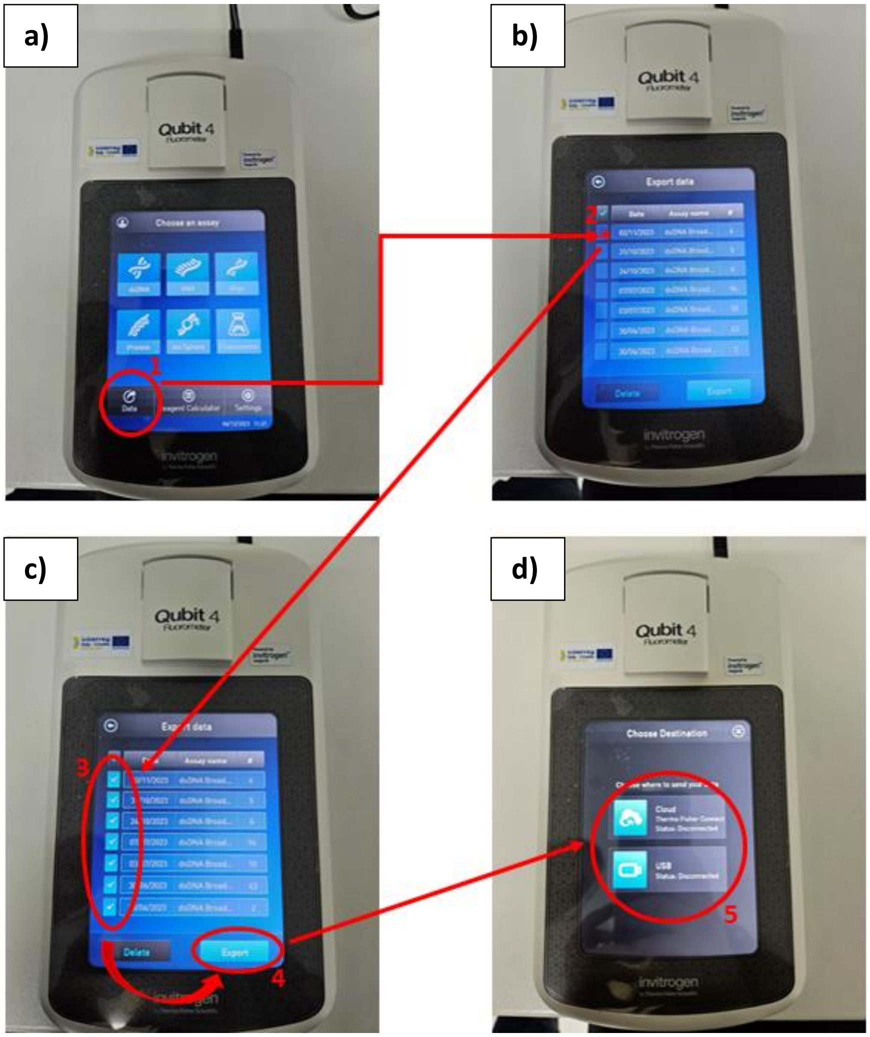


Figure 32. Qubit fluorometer: home screen (a), data export screen (b), data selected for export (c), data export destinations (d) (Author: L. Žuvić).

5.4. Measurement of DNA purity with an Implen NanoPhotometer

Note - The Implen Nano Photometer can also measure the concentration of extracted DNA, however the Qubit fluorometer provides more accurate concentration values, so in this case the Nano Photometer is only used to measure purity.

5.4.1. Mix the previously prepared (step: 5.1.1.) DNA samples in safe lock Eppendorf tubes briefly by vortexing or tapping with your finger (Figure 33) and then centrifuge briefly (a few seconds) using a mini centrifuge.

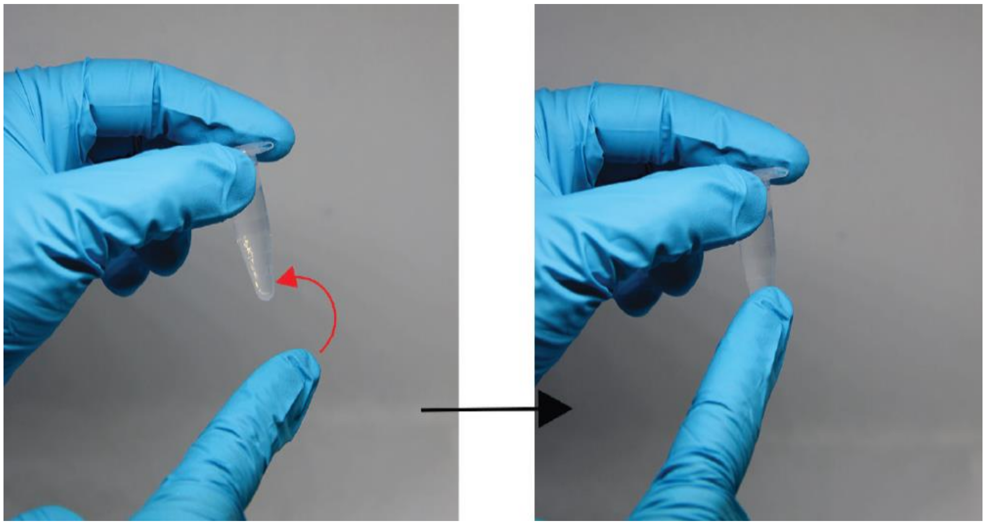


Figure 33. Mixing the extracted DNA sample by finger tapping (Author: L. Žuvić).

5.4.2. On the Implen NanoPhotometer N50 home screen, select the Nucleic Acids icon (Figure 34).

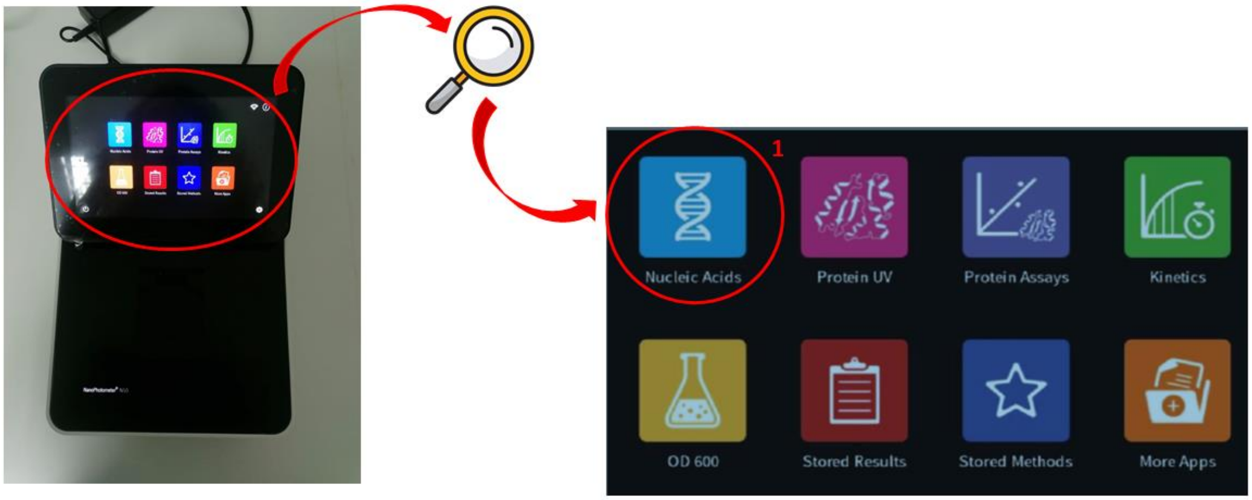
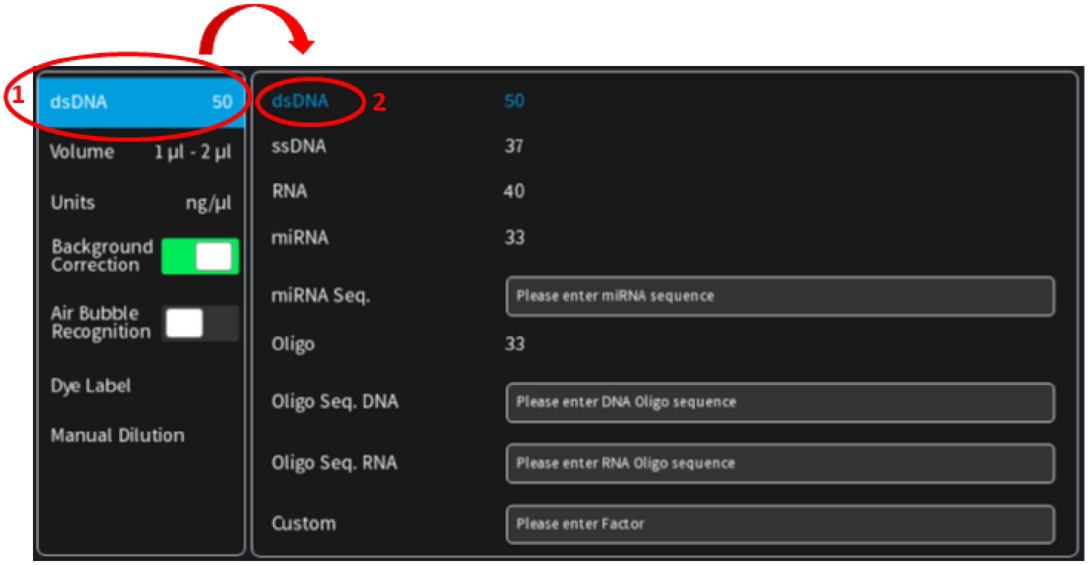


Figure 34. Implen NanoPhotometer N50: home screen with icons (Author: L. Žuvić).

5.4.3. To select the type of nucleic acid whose purity is to be measured, click on dsDNA. A list with the available options opens on the right-hand side of the screen: dsDNA, ssDNA, RNA, miRNA, miRNA Sequence, Oligo, Oligo Sequence DNA, Oligo Sequence RNA and Custom. Select dsDNA (Figure 35).

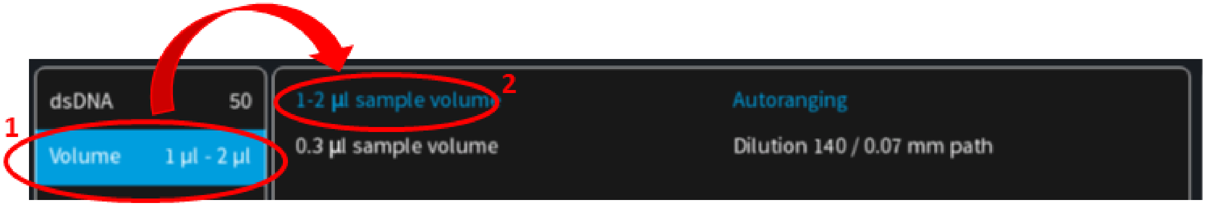


dsDNA 50
Volume 1 µl - 2 µl
Units ng/µl
Background Correction
Air Bubble Recognition
Dye Label
Manual Dilution

dsDNA 50
ssDNA 37
RNA 40
miRNA 33
miRNA Seq. Please enter miRNA sequence
Oligo 33
Oligo Seq. DNA Please enter DNA Oligo sequence
Oligo Seq. RNA Please enter RNA Oligo sequence
Custom Please enter Factor

Figure 35. Implen NanoPhotometer N50: screen with a list of nucleic acids available for selection (Author: L. Žuvić).

5.4.4. Select the volume of the DNA sample (1-2 µl) (Figure 36) to be applied to the sample illumination window (Figure 37).



dsDNA 50
Volume 1 µl - 2 µl

1-2 µl sample volume Autoranging
0.3 µl sample volume Dilution 140 / 0.07 mm path

Figure 36. Implen NanoPhotometer N50: part of the screen for selecting the sample volume (Author: L. Žuvić).

Figure 37. Implen NanoPhotometer N50: sample illumination window (Adapted from: NanoPhotometer NP80/N60/N50/C40 User manual).

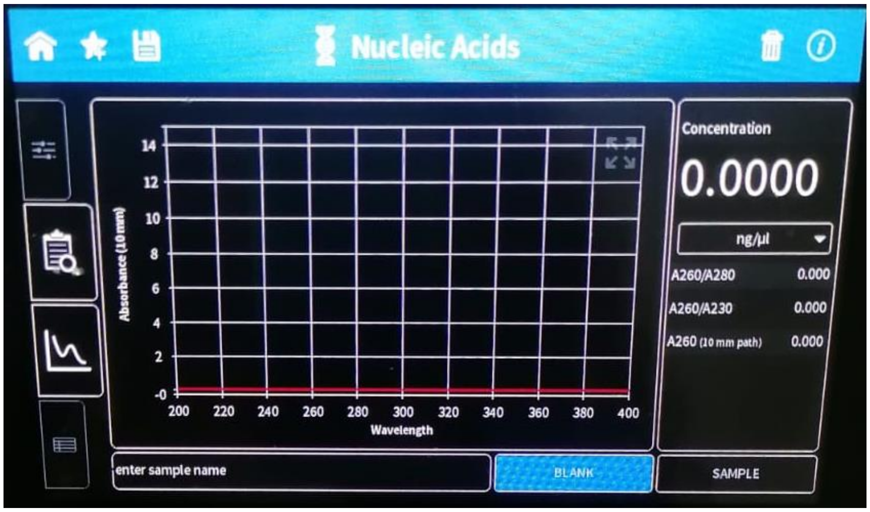
5.4.5. Pipette (1-2 µl) the AE buffer with which the DNA was eluted (Figure 38a) (step: 4.1.11.) onto the sample illumination window (Figure 38b) to calibrate the device. Close the sample illumination window and press BLANK to start the calibration (Figure 38c).

dsDNA 50
Volume 1 µl - 2 µl
Units ng/µl
Background Correction
Air Bubble Recognition
Dye Label
Manual Dilution

dsDNA 50
ssDNA 37
RNA 40
miRNA 33
miRNA Seq. Please enter miRNA sequence
Oligo 33
Oligo Seq. DNA Please enter DNA Oligo sequence
Oligo Seq. RNA Please enter RNA Oligo sequence
Custom Please enter Factor
enter sample name

Figure 38. AE buffer (a), Implen NanoPhotometer N50: Application of the AE buffer to sample illumination window (b), screen for calibration and settings (c) (Author: L. Žuvić).

5.4.6. When the calibration is complete, a screen appears with the concentration (ng/µl) and purity (A260/A280 and A260/A230) values of 0.000 (Figure 39). Open the sample illumination window and clean it with a lint-free laboratory wipe (Figure 40).



Concentration
0.0000
ng/µl

A260/A280 0.000
A260/A230 0.000
A260 (10 mm path) 0.000

Figure 39. Implen NanoPhotometer N50: display of the screen after calibration (Author: L. Žuvić).

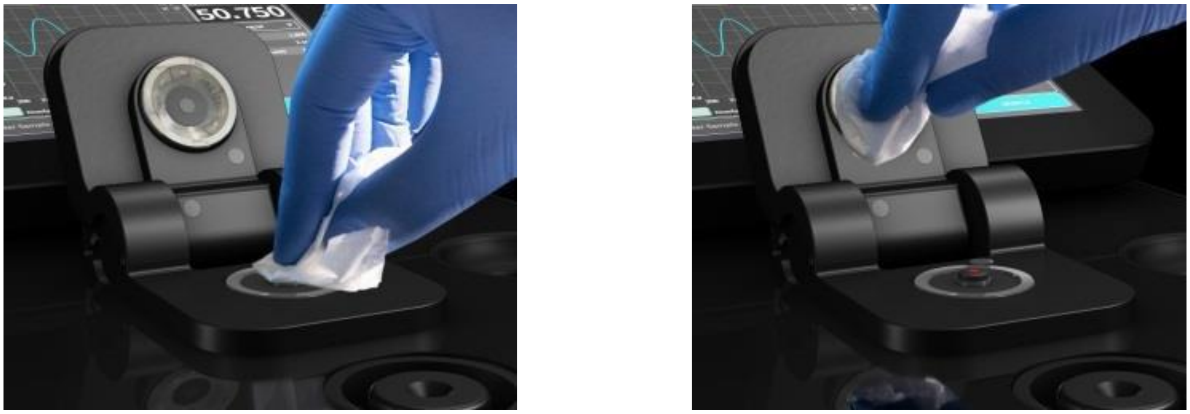


Figure 40. Cleaning the sample illumination window with a laboratory wipe (Reused from: NanoPhotometer NP80/N60/N50/C40 User manual).

5.4.7. Apply the DNA sample (1-2 µl) whose purity is to be measured to the cleaned sample illumination window in the same way as the AE buffer. Close the sample illumination window and press the enter sample name field on the screen displayed after calibration to enter the name of the DNA sample whose purity is to be measured (Figure 41a). Press SAMPLE to start the measurement (Figure 41a). Once the reading is complete, the results of the measured purity (A260/A280 and A260/A230) and concentration (ng/µl) are displayed on the screen (Figure 41b).

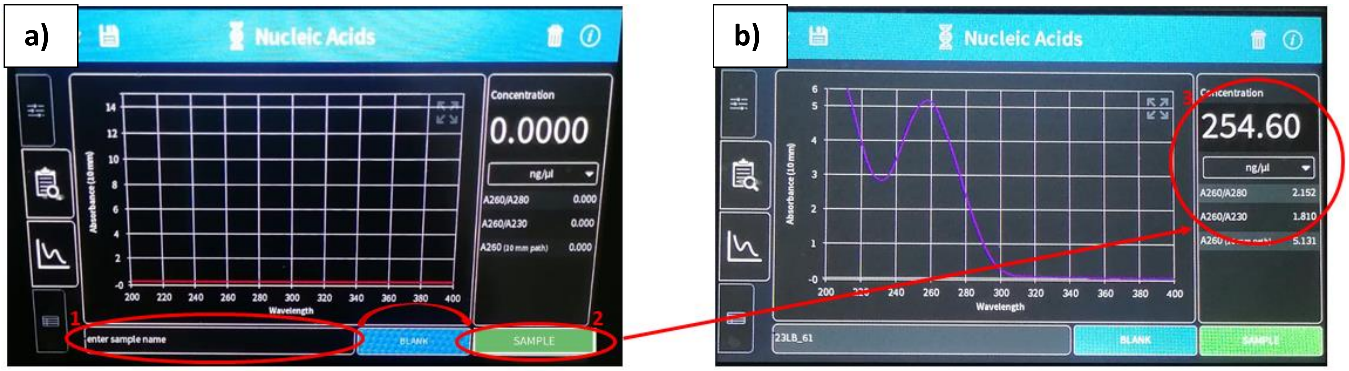


Figure 41. Implen NanoPhotometer N50: display of the screen after calibration (a) and screen with displayed results after reading the sample (b) (Author: L. Žuvić).

5.4.8. Repeat the procedure from step 5.4.7. for all DNA samples whose concentration is to be measured.

Note - before applying a new DNA sample to the sample illumination window, it should be cleaned with a laboratory wipe. On the screen, in the sample name entry field, the sample number must be changed to match the DNA sample applied to the sample illumination window.

5.5. Measurement of DNA purity with an Implen NanoPhotometer - saving and exporting data

5.5.1. On the Implen NanoPhotometer N50 home screen, select the Stored Results icon (Figure 42a). On the Stored Results screen that appears, press the data file to be exported (example: BFT_209_211_212_213_215_216_KIT.xlsx) and select the Copy to option (Figure 42b) and then the USB Flash Drive option to export the data to a USB by selecting Copy to (Figure 42c).

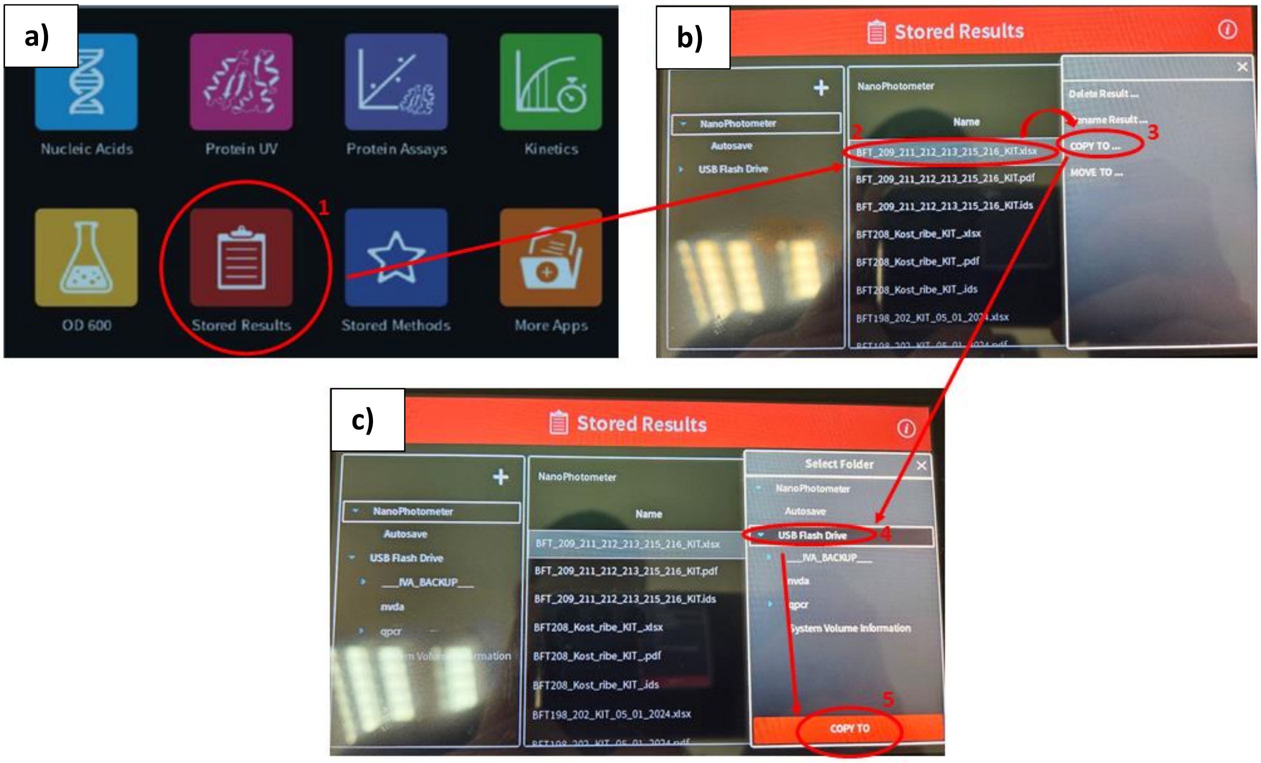


Figure 42. Implen NanoPhotometer N50: home screen (a), Stored Result screen with the data file to be exported (b) and export of selected data (c) (Author: L. Žuvić).

6. References

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