Biodiversity Genomics Europe

Biodiversity Research Integrating barcoding, Genomics and High-throughput Technologies

BRIGHT

TRAINING HANDBOOK

DRAFT

BGE joint network training

Łódź, Poland, 3-7 Feb 2025

Funded by the European Union
Schweizerische Eidgenossenschaft Confédération suisse Confederazione Svizzera
UK Research and Innovation

Training venue:

University of Lodz
Faculty of Biology and Environmental Protection
Department of Invertebrate Zoology and Hydrobiology

Banacha 12/16, 90-237 Łódź

Organizers:

UniLodz Polish Barcode of Life team(PoLBOL):

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Piotr Gadawski
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PoL BOL Polish Barcode of Life
Biodiversity Genomics Europe
iBOL EUROPE
international BARCODE OF LIFE
eRGA EUROPEAN REFERENCE GENOME ATLAS
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Thanks to the synergy of DNA barcoding, genomics methods and high-throughput sequencing technologies, recognition of biodiversity from individuals through populations to species levels became available for everyone. The workshop will provide practical training and theoretical knowledge on using modern molecular tools in a wide aspect of biodiversity studies.

The workshop is organised by the members of the Biodiversity Genomics Europe project founded by the European Union program Horizon Europe.

The workshop will be transmitted online using:

Microsoft Teams Need help?

Join the meeting now

Meeting ID: **321 726 784 097**

Passcode: **no95wH6C**

Channel:

0400-BGE-course_Feb2025 | General | Microsoft Teams

If you have any problems with online meetings contact:

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Content:

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Our Instructors:

Theory and general instructors:

Karolina Bącela-Spychalska (KBS), University of Lodz, Poland
Thomas Fullerton Brown (TB), Leibniz Institute of Zoo and Wildlife Research, Germany
Andrea Desiderato (AD), University of Lodz, Poland
Michał Grabowski (MG), University of Lodz, Poland
Piotr Gadawski (PG), University of Lodz, Poland
Marcin Kamiński (MK), Museum & Institute of Zoology, Polish Academy of Sciences, Poland
Tomasz Mamos (TM), University of Lodz, Poland
Joana Paupério (JP), European Molecular Biology Laboratory, Hinxton, UK
Lyndall Pereira da Conceicoa (LPC), Wellcome Sanger Institute, UK
Tomasz Rewicz (TR), University of Lodz, Poland
Felix Shaw (FS), Earlham Institute, UK
Igor Siedlecki (IS), University of Warsaw, Poland
Amrita Srivathsan (AS), National History Museum, Germany
Grzegorz Tończyk (GT), University of Lodz, Poland
Marta Sobalska-Kwapis (MSK), University of Lodz, BioBank, Poland

Laboratory:

leading: Gabriela Karlik (GK), University of Lodz
leading: Tomasz Mamos (TM), University of Lodz
Karolina Biniek (KB), University of Lodz
Wiktoria Cechowicz (WC), University of Lodz
Piotr Gadawski (PG), University of Lodz
Serena Mucciolo (SM), University of Lodz
Tomasz Rewicz (TR), University of Lodz

Computing:

leading: Thomas Fullerton Brown (TB), Leibniz Institute of Zoo and Wildlife Research, Germany
leading: Amrita Srivathsan (AS), National History Museum, Berlin
Andrea Desiderato (AD), University of Lodz
Tomasz Mamos (TM), University of Lodz

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Training schedule:

Lectures (room 501/502)
Practical classes in wet laboratory (room 4.4)
Practical classes outside the main building or using computers in room 501/502

Monday, 3 Feb 2025

9:00-10:30	Welcome and morning coffee Introduction of attendees and Instructors
10:30-10:45	Coffee break
10:45-12:00	DNA barcoding and BGE project (MG)
12:00-13:15	Lunch break
13:15-14:30	Planning DNA-based biodiversity study (PG)
14:30-14:45	Coffee break
14:45-16:30	eDNA in biodiversity assessment (KBS, AD)
16:30-16:45	Coffee break
16:45-18:00	DNA Isolation, amplification and quality control (TM)

Tuesday, 4 Feb 2025

9:00-10:30	Introduction to work in molecular laboratory (SM) Laboratory: DNA isolation for barcoding
10:30-10:45	Coffee break
10:45-12:00	BOLD database, sequence ID (TR)
12:00-13:15	Lunch break
13:15-14:00	Collecting material in practice (PG, GT)
14:00-14:15	Coffee break
14:15-16:30	Laboratory: High Molecular Weight (HMW) DNA isolation
16:30-16:45	Coffee break
16:45-18:00	The BIOSCAN UK Project (LPC) Barcoding with Nanopore (TM)

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Wednesday, 5 Feb 2025

9:00-12:00	Laboratory: DNA amplification for Nanopore sequencing, QC of HMW DNA
12:00-13:15	Lunch break
13:15-14:30	DNA barcoding for fungi (IS)
14:30-14:45	Coffee break
14:45-18:00	Laboratory: DNA pooling, ONT library preparation, QC

Thursday, 6 Feb 2025

9:00-10:30	Large-scale barcoding using Nanopore sequencing (AS)
10:30-10:50	Coffee break
10:45-12:00	Computing: ONTbarcoder Sequence ID (AS)
12:00-13:15	Lunch break
13:15-14:30	Museomics 101: How to harvest historical DNA from zoological collections? (MK)
14:30-14:45	Coffee break
14:45-16:30	FAIR data management (JP, FS)
16:30-16:45	Coffee break
16:45-18:00	Reference genomes, Galaxy server (TB)

Friday, 7 Feb 2025

9:00-12:00	Computing: Genome assembly using Galaxy (TB), including coffee break
12:00-13:15	Lunch break
13:15-14:30	Visit in Biobank UniLodz, library preparation for Illumina (MSK)
14:30-14:45	Coffee break
14:45-16:30	Computing: Genome assembly using Galaxy (TB)
16:30-16:45	Coffee break
16:45-18:00	Citizen Science for biodiversity research (KBS) Summary of the BRIGHT training

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Brief description of particular sessions, according to schedule:

Monday

Introduction of attendees and Instructors

A short session in which we will introduce ourselves (including participants) and describe briefly the workshop and the hosting institution (Faculty of Biology and Environmental Protection of the University of Lodz).

DNA barcoding and BGE project (Michał Grabowski)

My lecture will introduce the audience to DNA barcoding and derived methods such as (meta)barcoding and eDNA. I will start with the general problems of biodiversity assessment and explain why we need to solve them. I will also briefly present the history and rationale behind the concept of DNA barcoding and list issues it can help solve (and where it can't) - including the controversial issue of turbo-taxonomy. I will illustrate my talk with appropriate case studies. Finally, I will present the goals of the EU Horizon Europe *Biodiversity Genomics Europe* project, under which this workshop is being held.

Planning DNA-based biodiversity study (Piotr Gadawski)

During the presentation, I will discuss planning and organising a field trip to collect material suitable for DNA-based biodiversity assessment. The presentation will be devoted to practical aspects of planning such a trip, activities during the field trip and how we should treat samples after the field trip. I will also bring examples of well-organised expeditions for DNA-based analysis.

eDNA in biodiversity assessment (Karolina Bącela-Spychalska, Andrea Desiderato)

The three main topics of this panel will be what environmental DNA (eDNA) is, how to sample it, and what to do with it. We will introduce you to the eDNA and how it is revolutionising biodiversity conservation study through its limitations and how the technology is advancing. All you want to know to start working with eDNA: Bruce K, et al. (2021) A practical guide to DNA-based methods for biodiversity assessment. Advanced Books. https://doi.org/10.3897/ab.e68634

An experiment and review testing different methods of sampling eDNA: Kirtane, A., et al. (2024), How, What, and Where You Sample Environmental DNA Affects Diversity Estimates and Species Detection. Environmental DNA, 6: e70042. https://doi.org/10.1002/edn3.70042

An example of new technologies and applications to remote sensing with eDNA: Kirchgeorg S., et al.. *Environmental Science & Technology* 2024 *58* (37), 16410-16420; DOI: 10.1021/acs.est.4c05595

DNA Isolation, amplification and quality control (TM)

We will get familiar with the common techniques used in molecular biology including different techniques of DNA isolation, principles of polymerase chain reaction and quality control of isolated DNA and amplified markers. The gained knowledge will be used in following practicals.

Tuesday

Introduction to work in the molecular laboratory (Serena Mucciolo)

The presentation will thoroughly introduce working in a molecular laboratory, highlighting essential practices, protocols, and key safety measures. It will cover proper handling and storage of chemicals, safe and efficient waste disposal procedures, and using personal protective equipment (PPE) to ensure a safe work environment. Additionally, the importance of maintaining a clean and organised workspace, adhering to lab-specific guidelines, and following best practices to minimise contamination and ensure reliable experimental outcomes will be emphasised.

Laboratory: DNA isolation for barcoding

Practical work in molecular laboratory on quick, high-throughput DNA isolation method employing Chelex. The isolation will be performed on different species of pollinators.

Protocol based on the: Casquet, J., et al. 2012. Chelex without boiling, a rapid and easy technique to obtain stable amplifiable DNA from small amounts of ethanol-stored spiders. Molecular ecology resources 12, 136-141.

Other quick techniques of high-throughput isolation:

HotSHOT DNA isolation: Truett, G. E., et al. (2000). Preparation of PCR-Quality Mouse Genomic DNA with Hot Sodium Hydroxide and Tris (HotSHOT). BioTechniques, 29(1), 52–54.

Lysis C protocol with references therein.

BOLD database, sequence ID (Tomasz Rewicz)

An overview of the Barcode of Life Datasystems (BOLD) database and training on how to get your data to it (https://boldsystems.org/).

Collecting material in practice (PG, GT)

During the short visit in the botanical garden of the Faculty of Biology and Environmental Protection we will introduce methods of sampling using a Malaise Trap and kick net sampling in artificial ponds and basic eDNA sampling methods.

Laboratory: High Molecular Weight (HMW) DNA isolation

Practical work in a molecular laboratory on isolating high molecular weight DNA emptying using a commercial kit. The isolation is performed on the Crustacea-Amphipoda species *Gammarus jazdzewskii*. The high molecular weight DNA is isolated using Monarch® HMW DNA Extraction Kit for Tissue. The entire protocol can be found here. The modified producer protocol for the purpose of this course can be found below.

The BIOSCAN UK Project (Lyndall Pereira da Conceicoa)

The BIOSCAN UK Project led out of the Wellcome Sanger Institute, United Kingdom is a five+ year ambition to investigate the species diversity and spatiotemporal patterns of 1 million arthropods. We complete 24-hour monthly Malaise trap collections across the UK using a partnership model, with over 50 different partners (e.g. nature reserves, national parks, environmental agencies) running trapping events and plating individual arthropods. The DNA from specimens was then non-destructively DNA extracted and COI DNA barcoded using a highly multiplexed approach on the PacBio Sequel IIe. We have generated COI data for over 100,000 specimens and created an interactive data resource that enables anyone to easily explore the results and evaluate what species are found where and how this changes over time.

Project webpage: https://www.sanger.ac.uk/collaboration/bioscan/

Barcoding with Nanopore (Tomasz Mamos)

During the session we will become familiar with the nanopore sequencing technology introduced by Oxford Nanopore Technologies and its utility in DNA barcoding. Technology firstly used for generation of long DNA reads, thanks to small scale devices and downscaling sequence depth, enabled DNA sequencing for small laboratories. Nowadays the technology is gaining recognition for a generation of high quality full length molecular markers like DNA barcodes used for species recognition. Usage of tagged primers allowed for multiplexing and scaling of barcoding experiments that can range from hundreds to ten of thousands barcoded specimens in one sequencing. One of most important studies in this topic for invertebrates was done by Amrita Srivathsan in work: ONTbarcoder and MinION barcodes aid biodiversity discovery and identification by everyone, for everyone. BMC Biol 19, 217 (2021).

Wednesday

Laboratory: DNA amplification for Nanopore sequencing, QC of HMW DNA

At this laboratory we will focus on the polymerase chain reaction performed using tagged primers and quality control employing gel electrophoresis and DNA quantification with Qubit fluorometer.

DNA barcoding for fungi (Igor Siedlecki)

In the last decade, most of the fungal barcoding relied on Sanger sequencing and the ITS barcode (https://doi.org/10.1139/gen-2016-0046,). There are a few known issues with the ITS barcode (e.g. trouble with intragenomic variation: https://doi.org/10.1016/j.fbr.2022.04.002; negative bias towards early diverging fungi: https://doi.org/10.1111/1755-0998.13540). Secondary DNA barcodes are increasingly implemented for groups where ITS does not provide sufficient precision (https://doi.org/10.1186/s43008-020-00033-z). Recently, next generation sequencing platforms are being used much

more often in fungal barcoding allowing to deal with some of the currently known obstacles (e.g. PacBio https://doi.org/10.1111/1755-0998.13663; Illumina https://bsapubs.onlinelibrary.wiley.com/ doi/full/10.1002/aps3.11508; Nanopore https://www.nature.com/articles/s41598-023-37016 -0). Importantly, species delimitation in Fungi remains a matter of discussion (a recent review on Basidiomycota: https://doi.org/10.1007/s13225-021-00479-5). UNITE (https://unite.ut. ee/) is an ITS rDNA reference database curated by experts, in which a Species Hypothesis (SH) concept for molecular taxa delimitation is being implemented (https://doi.org/10 .1093/nar/gky1022; https://www.mdpi.com/2076-2607/8/12/1910). Finally, the importance of molecular data is growing in conservation mycology (https://doi.org/10.1016/j.cub. 2021.06.083).

Laboratory: DNA pooling, ONT library preparation, QC

We will process the tagged amplicons obtained from previous laboratory exercise, learn DNA cleaning procedure employing magnetic beads, learn preparation of DNA libraries for sequencing and finally practice the work with different types of nanopore sequencing flowcells.

Thursday

Large-scale barcoding using Nanopore sequencing (Amrita Srivathsan)

Computing: ONTbarcoder Sequence ID (Amrita Srivathsan)

Amrita, a pioneer in the field, will guide us through the use of nanopore sequencing in large scale experiments and show as her software ONTbarcoder. Amrita most important work in this topic:

Meier, R., et al. 2015. $1 DNA barcodes for reconstructing complex phenomes and finding rare species in specimen-rich samples. Cladistics 2015 Vol. 32 Issue 1.

Srivathsan, A., Lee, L., Katoh, K., Hartop, E., Kutty, S. N., Wong, J., Yeo, D., Meier, R. 2021. ONTbarcoder and MinION barcodes aid biodiversity discovery and identification by everyone, for everyone. BMC biology 19, 217.

Srivathsan, A., et al. 2024. ONTbarcoder 2.0: rapid species discovery and identification with real-time barcoding facilitated by Oxford Nanopore R10.4. Cladistics 40, 192-203.

Museomics 101: How to harvest historical DNA from zoological collections? (Marcin Kamiński)

Due to the recent advances in DNA sequencing technology, museomics is becoming a commonly used tool for solving different biological problems on all taxonomic levels. Up to now, those techniques enable us to trace the genetic history of selected species up to 1 million years ago (https://www.nature.com/articles/s41586-021-03224-9). The lecture summarizes major trends concerning DNA extraction, library preparation and sequencing for purposes of museomics. Furthermore, remarks are given on challenges in bioinformatics linked with the process of sequence assembly (e.g., https://www.nature.com/articles/s41598-021-91896-8).

FAIR data management (Joana Paupério, Felix Shaw)

The FAIR Guiding principles for scientific data management and stewardship were defined in 2016 to provide guidelines to improve Findability, Accessibility, Interoperability and Reuse of data. During this session we will introduce you to the FAIR principles and the importance of metadata and good data management for data publication and reuse. We will also give you an introduction of the Data infrastructures and tools available for genomics data publication and access. One of these is the COPO (Collaborative OPen Omics) platform, which is a portal for researchers to describe, store and search data easily, using community standards and public repositories. In addition, we will focus on the data and metadata model for sequence data publication to the INSDC (International Nucleotide Sequence Database

Collaboration), that includes the National Centre to Biotechnology Information (NCBI), the DNA Data Bank of Japan (DDBJ) and the European Nucleotide Archive (ENA).

Reference genomes/ galaxy (Thomas Fullerton Brown)

The increased accessibility and scalability of long-read sequencing has led to a recent explosion in the number of high quality *de novo* genome assemblies produced across the tree of life. These high quality reference genomes facilitate in depth research into the genetic mechanisms driving species adaptation, but also assist conservation efforts and management decisions. Initiatives such as the Earth Biogenome Project (EBP), European Reference Genome Atlas (ERGA) and Vertebrate Genomes Project (VGP) are coordinated efforts to produce reference genomes for all species on the global, continental and taxonomic scale, respectively.

Processing and managing the vast quantities of data requires robust, reusable and reproducible bioinformatic pipelines that can be executed on diverse datasets, organisms and infrastructures. Here, we will make use of the free-to-use, open-source compute infrastructure provided by the Galaxy Project to construct *de novo* genome assemblies from scratch.

The Reference Genome module will introduce the datatypes, algorithms and quality metrics determining what defines a high quality reference genome, with practical tasks accompanying every step. Ultimately you will gain an understanding of the current status of genomes published by researchers globally, how to determine the quality of a genome assembly and produce a chromosome-scale genome assembly using long- and short-read sequencing data.

References:

EBP: https://doi.org/10.1073/pnas.2115635118

ERGA and reference genomes for conservation: https://doi.org/10.1016/j.tree.2021.11.008

VGP and Galaxy Pipelines: https://doi.org/10.1038/s41587-023-02100-3

The Galaxy Project: https://doi.org/10.1093/nar/gkae410

Friday

Computing: Practical on genome assembly using Galaxy (Thomas Fullerton Brown)

During this session we will learn how to work with genomic data on the Galaxy server.

Visit in Biobank UniLodz, library preparation for Illumina (Marta Sobalska-Kwapis)

We will visit the local Biobank, learn how the biological samples are being stored, registered and shared. Additionally we will visit the sequencing facility within the Biobank, equipped with various Illumina sequencing machines and learn about their applications in biodiversity research.

Citizen Science for biodiversity research (Karolina Bącela-Spychalska)

In the era of dramatic biodiversity decline, there is a need to accelerate the discovery and monitoring of wildlife. Citizen Science (CS) can help in collecting and managing data on the taxa occurrence and population condition. Molecular methods can be easily implemented in the research involving CS. Here, we would like to present the examples of activities within the BGE project including CS and to show how we may attract, motivate and stay in touch with the non-scientists involved in our research.

Protocols

Below are protocols used in the local PolBOL laboratory in University of Lodz, they are based on the producer protocoles or linked publications and modified to fit scale or needs of the PolBOL laboratory. However, online there is a rich database of protocols for molecular biology, below some useful links:

https://www.protocols.io/

https://iboleurope.org/molecular-biology/

https://www.erga-biodiversity.eu/resources-1

Chelex DNA extraction protocol

Material needed: PCR plate/ stripe with coverings; Chelex 100 resin; ultra-pure H_2O, Proteinase K, 96% EtOH, forceps, magnetic agitator, thermocycler

- Fill the wells in a plate/stripe with 5 µl of 96% ethanol to prevent the material from being moved by static electricity in further steps
- Carefully remove 1–2 legs from a specimen with clean forceps
- Pick up the leg(s) and place them at the bottom of the first well
- Each time disinfect the forceps with 96% ethanol or in a flame
- After all the samples are prepared incubate at room temperature and ensure that all remaining ethanol has evaporated

	1 individual	1 plate	
Chelex	0.015 g	1.59 g	
UP H_2O	0.15 ml	15.9 ml	
Proteinaze K	10 µl	960 µl	

- Prepare the Chelex solution using following ingredients:
- Place a bottle containing a Chelex solution on a magnetic agitator and mix
- Pipet 160 µl of the solution to each well with the material
- Seal the plate and incubate it at 55 C for 6 hours
- Store the plate in a -20°C freezer.

High molecular weight DNA isolation

Part 1: TISSUE LYSIS

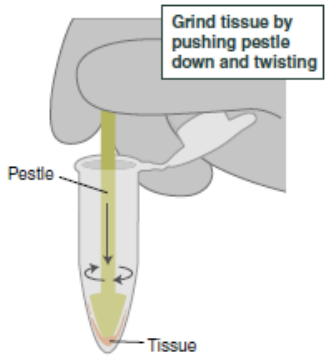
1. Prepare a master mix of HMW gDNA Tissue Lysis Buffer (600 ul) and Proteinase K (20ul) according to the number of samples that will be processed.

2. Transfer the desired amount of tissue (10-25 mg for most tissues) to a Monarch Pestle Tube (marked: **group number + 1**, example: Gr.1 1 Gr.2 1….). Place on ice.

3. Briefly spin sample (2-3 seconds) in a benchtop minicentrifuge to collect all tissue material at the bottom of the tube.

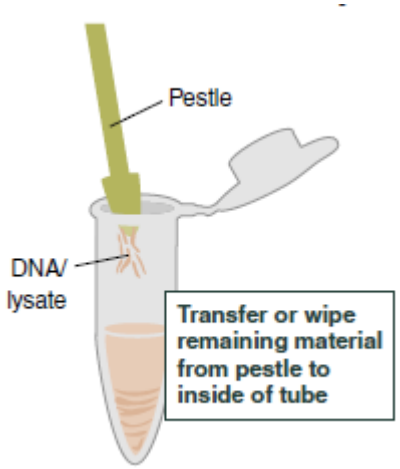
4. Homogenization:

a. Use the pestle to grind the sample within the pestle tube; leave the pestle in the tube.



b. Using a wide bore pipette tip, add 600 µl of the lysis master mix to the sample.

c. Ensure there is no tissue material remaining on the pestle, then discard the pestle.



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d. Using the wide-bore tip, pipette up and down a few times to homogenize the tissue lysate to ensure rapid, complete lysis. Discard the pipette tip.

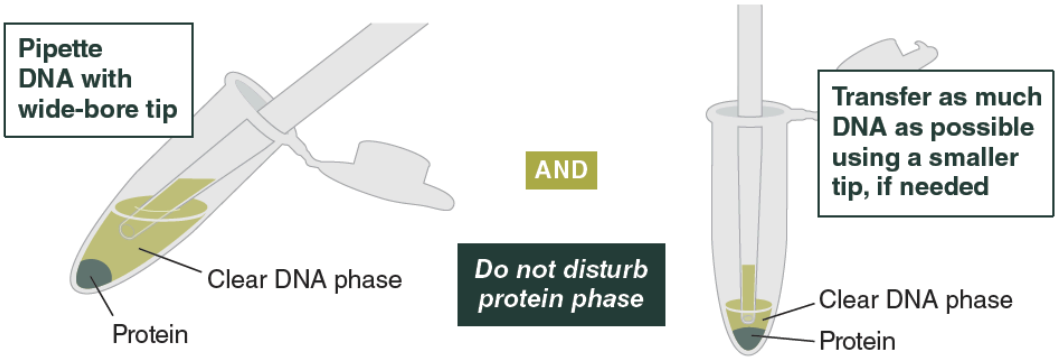
5. Incubate at 56°C for 45 minutes in a thermal mixer with agitation. If desired, samples can be stored at 4°C overnight after this incubation

6. Add 10 µl RNase A and mix by inverting 5-10 times. Incubate for 10 minutes at 56°C with agitation.

7. Add 300 µl of Protein Separation Solution and mix by inverting for 1 minute.

8. Centrifuge for 15 minutes at 16,000 x g.

9. Using a 1000 µl (Low Input: 200 µl) wide-bore pipette tip, transfer the upper phase containing the DNA (large, clear phase) to a labeled Monarch 2 ml Tube (marked: **group number + 2**, example: Gr.1 2 Gr.2 2….).

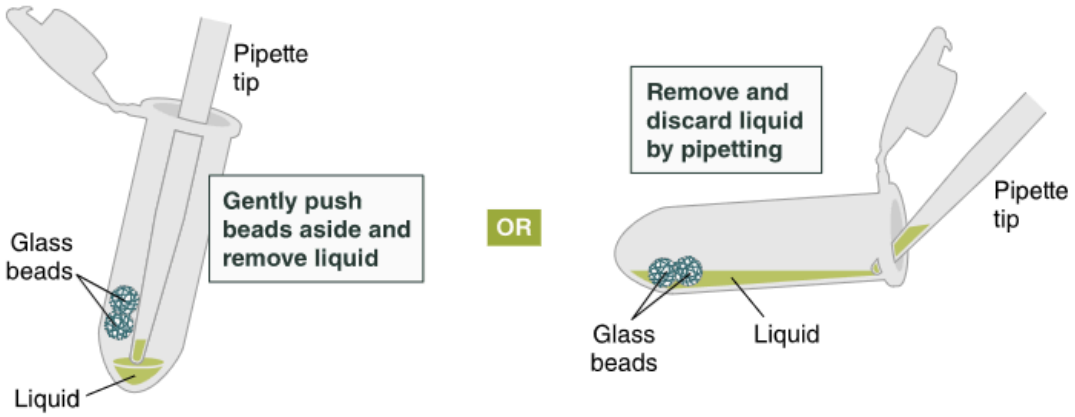


Part 2: HMW gDNA BINDING AND ELUTION

1. Using clean forceps, add 2 DNA Capture Beads to each sample, which should be contained in a Monarch 2 ml Tube.

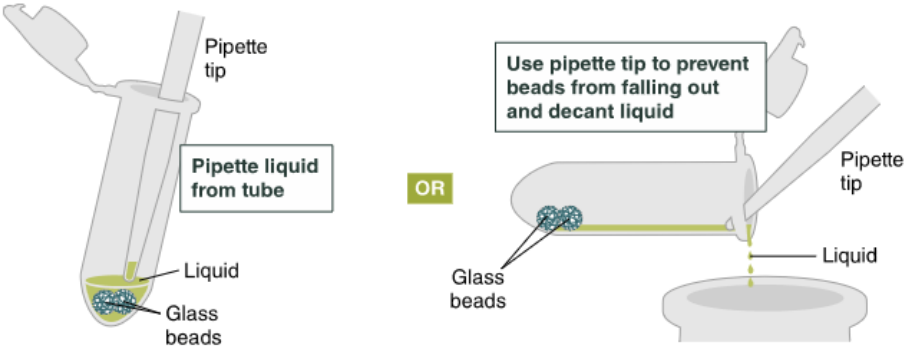
2. Add 550 µl isopropanol, close the cap, and mix inverting slowly gently by hand 25-30 times (5 minutes) to attach DNA to the beads.

3. Remove liquid by pipetting. Avoid removing any of the gDNA wrapped around the glass beads. For optimal DNA solubility, avoid letting the bound DNA dry out on the beads during this and the following steps; add the next buffer quickly. Keeping tube upright, insert pipette tip and gently push beads aside to remove liquid, or tilt the tube almost horizontally and remove liquid from the top of the angled tube.

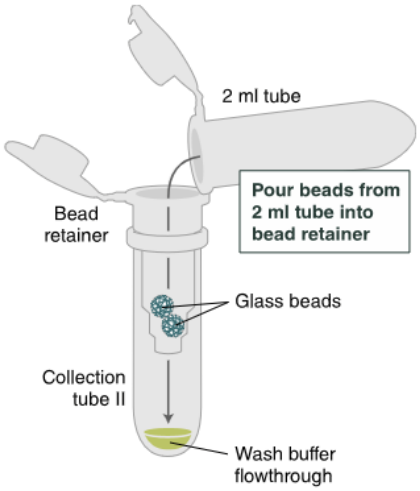


4. Add 500 µl gDNA Wash Buffer, close the cap and mix by inverting the tube 2-3 times. Remove the wash buffer as described in the previous step.

5. Repeat the wash in Step 4, and remove the wash buffer by pipetting. Alternatively, the buffer can be removed by decanting: position a pipette tip at the top of the angled tube to prevent the beads from falling out. It is not necessary to remove all the gDNA Wash Buffer at this point.

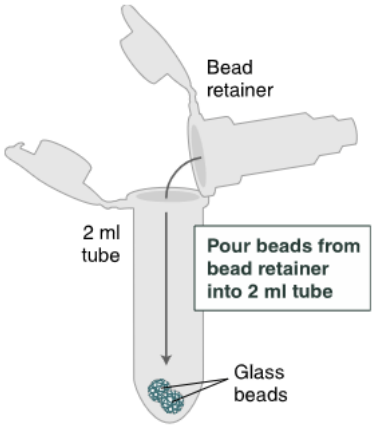


6. Place a labeled bead retainer into a Monarch Collection Tube II (marked: **group number + 3**, example: Gr.1 3 Gr.2 3….). Pour the beads into the bead retainer and close the cap. Discard the used Monarch 2 ml Tube.

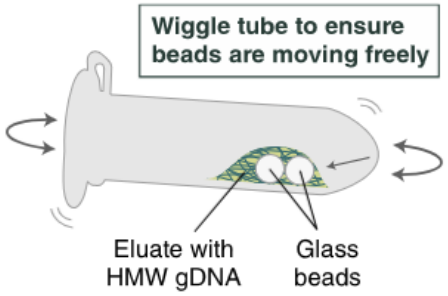


7. Pulse spin (≤ 1 second) the sample in a benchtop minicentrifuge to remove residual wash buffer from the beads.

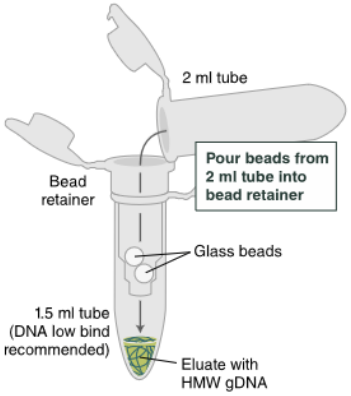
8. Separate the bead retainer from the collection tube, pour the beads into a new, labeled Monarch 2 ml Tube (marked: **group number + 4**, example: Gr.1 4 Gr.2 4….), and insert the used bead retainer into a labeled 1.5 ml microfuge tube (marked: **group number + 5**, example: Gr.1 5 Gr.2 5….) (DNA low bind recommended, not provided). Discard the used collection tube.



9. Immediately add 100 µl Elution Buffer II onto the glass beads and incubate for 5 minutes at 56°C in a thermal mixer with agitation.



10. Ensure bead retainer is inserted into the 1.5 ml microfuge tube. Pour eluate and beads into the bead retainer and close the cap.



11. Centrifuge for 30 seconds at 12,000 x g to separate the eluate from the glass beads. Discard the beads and retainer.

12. Pipette eluate up and down 5-10 times with a wide bore pipette tip and ensure any visible DNA aggregates are dispersed. Samples can be stored at 4°C for short term use (weeks) or -20°C for long term storage. The elution buffer (10 mM Tris, pH 9.0, 0.5 mM EDTA) is formulated for long term storage of gDNA.

Polymerase Chain Reaction (PCR) protocol for ONT sequencing on MinION flow cell

Require:

- plates with DNA isolates
- primers (forward and reverse)
- H_2O mol. biol. grade
- Dream Taq polymerase (preferably no dye)
- Plastics: all tips sets, 1.5 ml tubes, three new PCR plates

Procedure:

1. Thaw: isolates and reagents in RT, polymerase on ice.
2. Vortex and spin reagents and place on ice.
3. Prepare master mixes with **H_2O**, **Dream Taq and reverse primer** from respective tube, representing your plate number from MinION booking spreadsheet.

PCR master mixture for one plate:

Reagent	**1 spec.**	**For plate**
Dream Taq (x2)	6.5 ul	**689 ul**
H_2O	2.5 ul	**265 ul**
Reverse primer (2.5uM)	1 ul	**106 ul**
Sum per spec:	**10 ul**	**1580 ul**

4. Transfer **forward primers mix (2ul from 1.25uM)** from the primer plate on the new plates (on coolers), using multichannel pipette, the same set of tips for specific row on all plates.
5. Transfer respective volume (10 ul) of master mix.
6. Transfer **1ul amount of DNA** isolate (diluted in case of chelex), changing tip ich time.
7. Seal the plates with white silicone cap-mat, spin and place in the thermocycler.

PCR program :

1. 95° - 5 min.
2. 5x (95° - 50 sec.| 45° - 50 sec. | 72° - 1min.)
3. 35x (95° - 50 sec.| 51° - 50 sec. | 72° - 1min.)
4. 72° - 2 min
5. 12° - ∞

Pooling and cleaning of the PCR products with magnetic beads

Require:

- Sera-Mag Select reagent
- PCR plates with products
- Tips 10/200/1000 ul
- Eppendorf tubes 1.5ml
- Freshly made 85% ethanol (400ul, 340 ul EtOH+ 60ul UP-H_2O)
- H_2O molecular grade

Pooling

1. Pool 5ul from each well of the plate into a new 1,5 ml vial (signed: **original pool + group number**), mix well and centrifuge briefly (store in -20°C).
2. Transfer 200 ul into a new 1.5ml tube (signed: **DNA pool + group number**)(can be stored in the fridge overnight or in -20°C for long).

Cleaning

1. Resuspend/vortex Sera-Mag.
2. Add 140 ul Sera-Mag to the tube with 200 ul DNA sample pool.
3. Vortex the sample for 30-60 seconds, briefly spin and incubate at room temperature (RT) for 5-10 min.
4. Place the tube on a magnet rack for 5 min or until the beads have fully settled.
5. Transfer whole supernatant to the new 1.5 ml tube (signed: **super + group number**)(ca. 350 ul, for QC on gel).
6. With the tube on the magnet, wash twice gently with 180 µL 85% ethanol, do not disturb the bead pellet. Take care to aspirate as much wash solution as possible after the second wash.
7. Dry the beads for 3-6 min at RT to remove any residual ethanol. Watch the pellet, avoid the point of cracking (should be a bit shiny).
8. Remove from the magnet, add 35 µL of H_2O molecular grade, vortex gently for 30-60s to resuspend the beads and briefly spin.
9. Incubate for 5-10 min at RT to elute DNA.
10. Return to the magnet for 5 min or until the beads have fully settled.
11. Carefully aspirate the supernatant containing purified DNA and transfer to a new 1.5 ml tube (signed: **cleaned DNA + group number**).

Quality control (QC)

1. Run 1% small gel for original pool, supernatant and final, cleaned DNA. If a significant amount of oligos is visible in DNA, repeat cleaning.

2. Measure the DNA concentration on Qubit before library preparation (200 fmol is enough for MinION).

5. 5. Casting and running a gel protocol

The protocol uses a 1% TBE buffer in the gel electrophoresis step.

Prepare 1% gel using following ingredients:

- Put agarose to the glass
- Add TBE buffer
- Cover the glass with alu foil
- Put the glass with agarose and TBE to the microwave and heat (***) for 3 minutes
- After the agarose is diluted and the mixture is clear fill the missing amount of the liquid with distilled water if needed
- Check you are working on a level surface to cast a gel with uniform thickness
- Add SimplySafe and mix
- Pour the gel into the electrophoresis tank
- Check the agarose gel for bubbles
- Put the combs into the holder
- Wait until the gel hardens ~30 min

Run the gel:

- Pour the TBE buffer solution into the gel tank over your prepared gel until the liquid covers your gel, and the level is about 2-3 mm above your gel and remove the comb
- Carefully load a molecular weight ladder into the first lane of the gel
- Load your samples into the remaining wells of the gel
- Set the voltage to the desired electrophoresis voltage for your protocol.
- Set the desired time
- Run the gel until the dye line has travelled down about 75% the length of the gel.
- Place your gel on the transilluminator and visualise your result

MinION library preparation and sequencing protocol

Require:

› 100-200 fmol Amplicon DNA

› Pooled and quanted

› **AXP** - AMPure XP Beads
› **LA** - Ligation Adapter
› **LNB** - Ligation Buffer

› **SFB** - Short Fragment Buffer
› **EB** - Sequencing Buffer
› **FCT** - Flow Cell Tethe

› **SB** - Sequencing Buffer
› **FCF** - Flow Cell Flush
› **LIB** - Library Beeds

› **Ultra II End-prep Reaction Buffer**
› **Ultra II End-prep Enzyme Mix**
› **Quick T4 Ligase**

› 1.5ml Eppendorf DNA LoBind Tubes

› 0.2ml thin-walled PCR tubes

› Nuclease Free Water

› 500µl Freshly prepared 80% ethanol in 1.5µl Eppendorf

› **400µl** 100% ethanol
› **100µl** Nuclease Free Water

› Qubit

› Assay Tubes
› dsDNA Broad Spectrum Assay Kit

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eRGA European Reference Genome Atlas
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UK Research and Innovation

Procedure:

END-PREP:

Materials:

- Thaw **DNA** at room temperature
- Thaw **End-prep Reaction Buffer** on ice
- Thaw **End-prep Enzyme Mix** on ice
- **AXP**
- 0.2ml PCR tubes
- 1.5ml DNA LoBind tubes
- **Nuclease Free Water**
- 80% Ethanol

1. thaw the reagents and start point 3 + prepare ethanol for rinsing – Eppendorf 1,5 (EtOH)

- **400µl** 100% ethanol
- **100µl** Nuclease Free Water

500µl Total -> Vortex

2. Prepare **Ultra II End-prep Reaction Buffer** & **Enzyme Prep** and place on ice

- **Do Not Vortex End Prep Enzyme Mix** - flick, invert and spin
- Vortex **End-prep Reaction Buffer** and spin (*there may be sediment, mix well*)

3. Transfer 100-200 fmol amplicon DNA into 0.2ml PCR tube

- Vortex and spin DNA (calculated volume based on previous measurement) -> water first
- adjust to **50µl** with **nuclease-free water**
- flick and spin

4. Additionally, add:

- **7µl End-Prep Reaction Buffer** *(mix by pipetting)*
- **3µl End-Prep Enzyme Mix** *(mix by pipetting)*
  60µl Total
- Pipette mix, flick and spin

5. Thermal cycler - 5min @ 20°C, 5min @ 65°C *-> use a cap, and after incubation, spin the sample*

6. Spin and transfer **60µl** DNA sample to clean 1.5ml Eppendorf DNA LoBind Tubes

7. Heavily vortex **AXP** to resuspend and add **60µl** to DNA sample

- mix by flicking and spin

8. Incubate on Hula Mixer - 5min @ room temp *(from time to time gently flicking)*

-> prepare the reagents ***-> Adapter Ligation and Clean-up***
-> remove Qubit standards from the fridge

9. Spin down *(briefly)* the sample and pellet on a magnet until supernatant is clear and colourless.

- Keep the tube on the magnet, and pipette off the supernatant (**~120µl**)

10. Keep the tube on the magnet and wash the beads with **200µl** of freshly prepared 80% ethanol without disturbing the pellet. Remove the **200µl** ethanol using a pipette and discard

11. Repeat previous step

12. Spin down and place the tube back on the magnet.

- Pipette off any residual ethanol.
- Allow to dry for ~30 seconds, but do not dry the pellet to the point of cracking

13. Remove the tube from the magnetic rack and resuspend the pellet in **61 µl** nuclease-free water.

- Incubate for 2 minutes at room temperature *(gently flick)*

14. Pellet the beads on a magnet until the eluate is clear and colourless, for at least 1 minute

15. Remove and **retain 61µl** of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube

16. Quantify 1 µl of eluted sample using a Qubit fluorometer

It is possible to store this sample at 4°C overnight

Adapter Ligation and Clean-up:

Materials:

- **LA**
- **AXP**
- Thaw **LNB** at room temperature
- Thaw **SFB** at room temperature **or LFB**
  (To enrich for DNA fragments of 3 kb or longe)

- Thaw **EB** at room temperature
- **Quick T4 Ligase**
- 1.5ml Eppendorfs -> Qubit
- 3x 0.2ml PCR tubes
- 1.5ml Eppendorfs -> FIN POOL
- 1.5ml Eppendorfs -> Sequencing Mix

17. Spin down **LA** and **T4 Ligase**, place on ice

18. Prepare **LNB**

- Spin and mix by pipetting, then place on ice

19. Prepare **EB** and **SFB**

- Vortex, spin, place on ice

20. Into DNA Library (**60µl**), ad:

- **25µl LNB** *(mix by pipetting, before and after)*
- **10µl T4 Ligase** *(mix by pipetting)*
- **5µl LA** *(mix by pipetting)*
  100µl Total
- Pipette mix, spin briefly

21. Incubate - 10min @ room temperature

22. Heavily vortex **AXP** and add **40µl** to the reaction

- flick and spin

23. Incubate on Hula Mixer - 5min @ room temperature *(from time to time gently flicking)*

*-> prepare the reagents -> **Loading the Flongle Flowcell***
-> Qubit -> prepare 3x 0,2ml thin-walled PCR tubes (S1, S2, D)

24. Spin down the sample and pellet on a magnet.

- Keep the tube on the magnet, and pipette off the supernatant (**~70µl**)

25. Wash the beads by adding 250 µl Short Fragment Buffer (SFB) (or 250 µl Long Fragment Buffer (LFB))

- Remove Eppendorf from the magnetic rack
- Flick the beads to resuspend and briefly spin down

- Return the tube to the magnetic rack and allow the beads to pellet
- Remove the **250µl** supernatant using a pipette and discard

26. <u>Repeat the previous step</u>

27. Spin down and place the tube back on the magnet.

- Pipette off any residual supernatant.
- Allow to dry for ~30 seconds, but do not dry the pellet to the point of cracking

28. Remove the tube from the magnetic rack and resuspend pellet in **15µl EB**

29. Spin down and Incubate for 10 minutes at room temperature (For high molecular weight DNA, incubating at 37°C can improve the recovery of long fragments.

-> MinIon -> check pores
-> prepare a buffer for Qubit -> add day and mix by pipetting, quickly vortex -> Eppendorf 1,5 (name Q);
-> pipetting the buffer– (standards (S1,S2) 190µl, D - 199µl or less)
*-> prepare Primer Mix **point 34***

30. Pellet the beads on a magnet until the eluate is clear and colourless, for at least 1 minute

31. Remove and retain **15 µl** of eluate containing the DNA library into a clean 1.5ml Eppendorf DNA LoBind tube
(FIN POOL - place on ice)

Dispose of pelleted beads

32. Quantify **1µl** of eluted sample using a <u>Qubit fluorometer</u> *(incubate for 2 min)*

-> finish preparing standards (10µl -> 0.2ml thin-walled PCR tubes (S1, S2))
-> flick, and spin S1, S2, D– measurement of standards (S1 ~ 200; S2 ~ 20 000) and DNA

33. Make up library to **<u>12µl</u>** <u>at 35-50 fmol</u> with **EB** *-> (SEQ MIX ->, first add EB then DNA -> finish volume 12µl, mix by pipeting).*

Store the library on ice or at 4°C until ready to load.

<u>Loading the MinION Flowcell:</u>

Materials:

- Thaw FCT room temp
- Thaw SB room temp

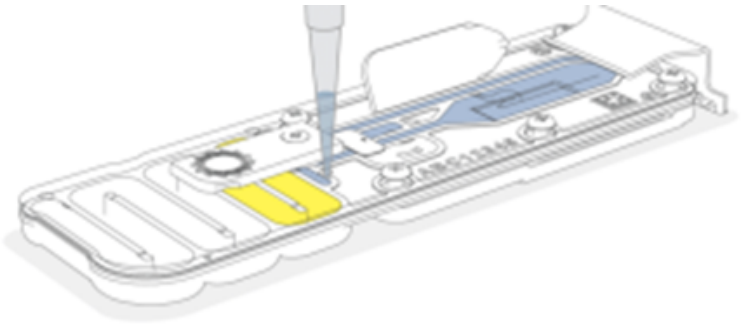
- Thaw FCF room temp
- Thaw LIB room temp
- 1.5ml Eppendorf -> Primer Mix

34. Prepare **SB**, **LIB**, **FCT** and **FCF**, vortex and spin

35. Create flow cell **Primer Mix** in a 1.5ml Eppendorf by adding:

Reagent	Volume per flow cell
Flow Cell Flush (FCF)	1,170 µl
Bovine Serum Albumin (BSA) at 50 mg/ml	5 µl
Flow Cell Tether (FCT)	30 µl
Total volume	**1,205 µl**

36. Slide the flow cell priming port cover clockwise to open the priming port.



37. After opening the priming port, check for a small air bubble under the cover. Draw back a small volume to remove any bubbles:
    1. Set a P1000 pipette to 200 µl
    2. Insert the tip into the priming port
    3. Turn the wheel until the dial shows 220-230 µl, to draw back 20-30 µl, or until you can see a small volume of buffer entering the pipette tip

Note: Visually check that there is a continuous buffer from the priming port across the sensor array.

38. Load **800 µl** of the priming mix into the flow cell via the priming port, avoiding the introduction of air bubbles. Wait for 5 minutes. During this time, prepare the library for loading.

39. In a new 1.5 ml Eppendorf DNA LoBind tube, prepare the library for loading:

Reagent	Volume per flow cell
Sequencing Buffer (SB)	37.5 µl
Library Beads (LIB) mixed immediately before use, or Library Solution (LIS), if using	25.5 µl
DNA library	12 µl
Total	**75 µl**

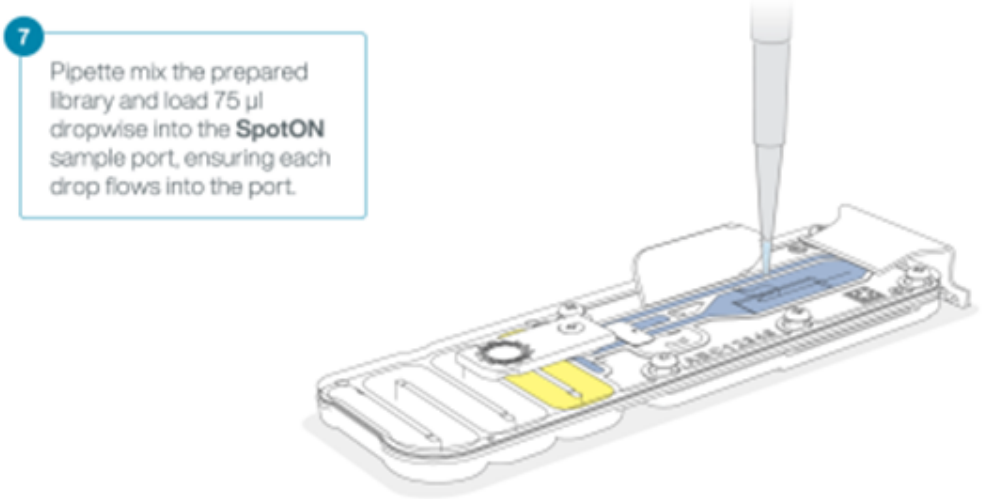
40. **Complete the flow cell priming:**

1. Gently lift the SpotON sample port cover to make the SpotON sample port accessible.

2. Load **200 µl** of the priming mix into the flow cell priming port (not the SpotON sample port), avoiding the introduction of air bubbles.

41. Mix the prepared library gently by pipetting up and down just prior to loading.

42. Add **75 µl** of the prepared library to the flow cell via the SpotON sample port in a dropwise fashion. Ensure each drop flows into the port before adding the next.



43. Gently replace the SpotON sample port cover, making sure the bung enters the SpotON port and closes the priming port.

44. Place the light shield onto the flow cell:

    1. Carefully place the leading edge of the light shield against the clip. Note: Do not force the light shield underneath the clip.

    2. Gently lower the light shield onto the flow cell. The light shield should sit around the SpotON cover, covering the entire top section of the flow cel.

45. Close the device lid and set up a sequencing run.

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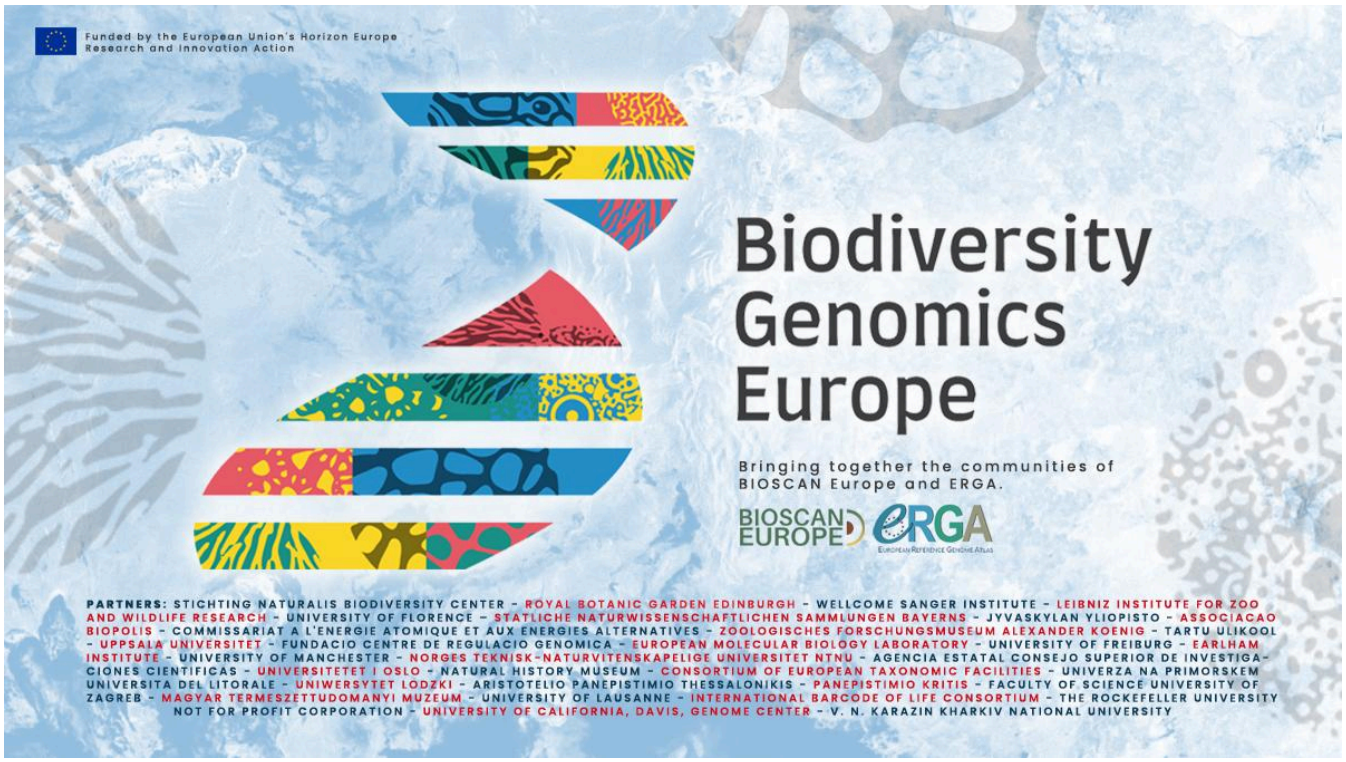

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