# Protocol for the EURGen-RefLabCap External Quality Assessment exercise 2024

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**APPENDICES**

## Disclaimer:

For the purposes of this document, this activity is referred to as an External Quality Assessment exercise (EQA). Currently, however, there are no harmonized standard methods for producing and analyzing results through whole-genome sequencing technologies. Therefore, this activity could instead be referred to as a Proficiency Test (PT).

# 1. Overview and objectives

External Quality Assessment (EQA) exercises are important tools to assess the capacity of laboratories to follow their own routine procedures and obtain accurate results. This assessment is done by comparing the achieved results with expected results produced by standard methods, and with results obtained by other laboratories performing the same type of analysis. The EURGen-RefLabCap EQA 2024 is a whole-genome sequencing (WGS) EQA focusing on bacterial species included in workstream 1 (WS1) (*Enterobacterales,* specifically *Escherichia coli* and *Klebsiella pneumoniae*) and workstream 2 (WS2) (*Acinetobacter baumannii* and *Pseudomonas aeruginosa*) of the EURGen-RefLabCap project.

The EURGen-RefLabCap EQA 2024 is coordinated as part of a contract with the European Health and Digital Executive Agency (HaDEA) on behalf of the General Directorate Health and Food Safety (DG SANTE) in close collaboration with European Centre for Disease Prevention and Control (ECDC) (SC 2019 74 01 – Service Contract for the provision of EU networking and support for public health reference laboratory functions for antimicrobial resistance in priority healthcare associated infections). This contract is carried out jointly by the leads of the contract, National Food Institute, Technical University of Denmark (DTU) and the co-contractor National Reference Laboratory for Antimicrobial Resistance (NRL-AMR), Statens Serum Institut (SSI) of Denmark.

The main objective of this EQA is to test and compare technical and analytical skills of national reference laboratories (NRLs) in Europe for resistome profiling and high-risk clone/plasmid identification of colistin and/or carbapenem-resistant *Enterobacterales* (CCRE), *Acinetobacter baumannii* (C/CRAb), and *Pseudomonas aeruginosa* (C/CRPa). After participation in the EQA, members of EURGen-RefLabCap project will be able to identify strengths and weaknesses in their WGS and bioinformatics analysis skills for those pathogens. Furthermore, they will gain experience that will qualify them to design and execute EQA exercises in their own national networks.

The EQA providers will compare the applied national bioinformatics pipelines used by NRLs for WGS-based detection of antimicrobial resistance (AMR) and other important genetic determinants, to verify that results are compatible with those obtained using ECDC and the European Food Safety Authority (EFSA) joint molecular typing platforms and aligned with European case definitions and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidance documents.

## 2. Introduction

The EQA 2024 is the third iteration of EURGen-RefLabCap EQAs and it focuses on WGS as well as *in silico* analyses, for bacterial typing and identification of genomic elements responsible for AMR in *Escherichia coli*, *Klebsiella pneumoniae, Acinetobacter baumannii* and *Pseudomonas aeruginosa.* Furthermore, the EQA 2024 aims at evaluating the methods used for handling and extracting DNA from live bacterial cultures. In the EQA 2024, four isolates belonging to the four bacterial species mentioned above are included.

For each strain of the above-mentioned bacterial species, laboratories are expected to handle and process two types of test materials, as described below (please note that signing up for each species separately is allowed):

- **Item 1: Live bacterial cultures** (referred to as "BACT") for which DNA extraction and purification, library-preparation, WGS, and *in silico* analyses must be performed. The cultures are provided as swabs in transport media.
- **Item 2: Pre-isolated DNA** (referred to as "DNA") for which library-preparation, WGS, and *in silico* analyses must be performed. Each vial contains a minimum of 125ng DNA.

When signing up for Item 1 (bacterial cultures) for one species, participation in Item 2 (pre-isolated DNA) is expected. If, for any reason, participants are unable to handle live cultures and perform DNA extraction, it will be possible to submit results only for Item 2 (pre-isolated DNA). Participants must perform WGS with either short-read or long-read sequencing technology, using the routine methods and protocols currently implemented in their laboratories.

Participating laboratories nominated for workstream 1 (WS1) will receive test materials (live bacterial cultures AND pre-isolated DNA) from two isolates for testing belonging to the species included in WS1. Participating laboratories nominated for both WS1 and workstream 2 (WS2) will receive test materials (live bacterial cultures AND pre-isolated DNA) from all four isolates for testing. Information on the methods used to extract the DNA and to generate the EURGen-RefLabCap EQA 2024 test sequence data is available in Appendix 1.

**Table 1**: Test material and sample codes in the EURGen-RefLabCap EQA 2024.

| Test strain ID | Material type | Test material ID |
|---|---|---|
| **EURGEN-2024-01** | BACT | EURGEN-2024-01_BACT |
| | DNA | EURGEN-2024-01_DNA |
| **EURGEN-2024-02** | BACT | EURGEN-2024-02_BACT |
| | DNA | EURGEN-2024-02_DNA |
| **EURGEN-2024-03** | BACT | EURGEN-2024-03_BACT |
| | DNA | EURGEN-2024-03_DNA |
| **EURGEN-2024-04** | BACT | EURGEN-2024-04_BACT |
| | DNA | EURGEN-2024-04_DNA |

# 3. Outline of the EURGen-RefLabCap EQA 2024

### 3.1.1. Comparison with the previous EQA

The EQA 2024 is conducted similarly to the EURGen-RefLabCap EQA 2023. The main difference is the participating laboratories will receive both live cultures and pre-isolated DNA for sequencing (instead of receiving only pre-isolated DNA). Similar to the EQA 2023, we (the EQA organizers) will again request that the participants share with us the raw sequencing data that they have generated. In contrast to the EQA 2023, we will request sequences generated from both "BACT" and "DNA" test materials. The quality of the sequencing data will be assessed, and the results will be shared with the participating laboratories.

## 3.2. Receipt of test material

In February 2024, contact persons from the EURGen-RefLabCap project received the pre-notification and instructions for registration of the EURGen-RefLabCap EQA 2024. Those who

registered their laboratory to participate in the EURGen-RefLabCap EQA 2024 will receive the test materials in May 2024. Participants who are unable to sequence the DNA and have requested the sequencing data corresponding to the test material will receive a separate email with instructions on how to download the data via the ScienceData platform (see Appendix 2).

Recipient laboratories are welcome to store the test material (live cultures and pre-isolated DNA) for confirmatory purposes, e.g., repeating the exercise after the expected results for the EQA are available to troubleshoot potential problems, to benchmark adjustments to their methods (e.g., repeating the exercise when new library preparation kits are implemented), and for reference purposes (e.g., if any of the test materials contain genetic determinants of importance for national diagnostics or surveillance purposes that could be useful as controls for routine procedures).

## 3.3. Handling and storage of the test materials

### 3.3.1. Handling and storage of live cultures (Item 1)

Live bacterial cultures are shipped as cotton swabs in transport media. Upon receipt, the laboratories can store the transport swabs at 5-25°C, if immediate processing is not planned. Within 48 hours of receiving the swabs, the participants are encouraged to subculture and prepare the stock bacterial cultures for storage in their strain collection (e.g., in a -80°C freezer). After subculturing, laboratories can proceed with the DNA extraction and sequencing.

### 3.3.2. Handling and storage of pre-isolated DNA (Item 2)

The pre-isolated DNA is provided as dehydrated DNA in Eppendorf tube. Each Eppendorf tube of the pre-isolated DNA contains a minimum of 125 ng of dehydrated genomic DNA.

If immediate sequencing is intended (within two days of receiving the test material), the pre-isolated DNA can be rehydrated upon arrival. The rehydrated DNA can preferably be stored in the refrigerator (4°C - 8°C), or alternatively at room temperature.

If sequencing of the samples is planned within the first 10 days of arrival of the shipment, dehydrated DNA may be stored in:

- a dry storage cabinet at room temperature (15-25°C), or

- a heat-sealed, moisture-barrier bag along with a silica gel desiccant pack, or
- the zip-lock bag in which they arrived along with the silica gel desiccant pack. If moisture starts to appear, the desiccant pack must be changed. The dried samples can be stored at room temperature.

If sequencing is planned after 10 days of arrival of the shipment, we recommend that you rehydrate the pre-isolated DNA and store it at -80ºC, or alternatively at -20ºC (to rehydrate the DNA, please see instructions below, section 3.3.3).

### 3.3.3. Rehydration of the pre-isolated DNA

Before use, the dehydrated DNA should be rehydrated:

- Add 50 µL nuclease free water or aqueous buffer to the dehydrated DNA;
- To make sure you dissolve all the DNA, pipette up and down and let the water/buffer slide down all the sides of the Eppendorf tube. The DNA was dried in a vacuum centrifuge, and it is possible that some of the DNA is stuck onto the walls of Eppendorf tube. Pipette carefully to avoid shearing the DNA;
- Incubate the Eppendorf tubes at room temperature for 15 minutes to allow complete rehydration;
- Mix the suspension by gently tapping with the finger or pipetting up and down using wide-bore pipette tip. Please do not vortex the tube otherwise you risk shearing the DNA;
- The rehydrated DNA can now be used directly in downstream applications. We recommend storing the leftover DNA at -80ºC, or alternatively at -20ºC.

**Optional**: The quality and integrity of the rehydrated DNA can be evaluated by agarose gel electrophoresis. The amount of DNA supplied in each tube is sufficient to run a small fraction on agarose gel.

## 3.4. Sequencing of the pre-isolated DNA

The participants may choose to sequence the DNA by short-read or long-read sequencing technology using the routine methods and protocols currently implemented in their laboratories.

## 3.5. Processing of sequence data

The participating laboratories will be requested to report results from WGS-based prediction of multi-locus sequence types (MLST), detection of plasmid replicon types, detection of antimicrobial resistance genes (ARGs) and chromosomal point mutations (PMs) mediating AMR towards clinically important antimicrobials, and *in silico* prediction of AMR profiles (Table 2).

The participants may decide to analyse all the test materials they have received, or a selection of those materials. The participants may also decide to analyse a selection of the tests mentioned in Table 2, i.e., may report the analysis of ARGs or MLST only, or may proceed with reporting all the components mentioned in Table 2. One set of results for each test material may be submitted for evaluation. However, please note that the if the results for Item 1 (BACT samples) is submitted, it is also expected that the results for Item 2 (DNA samples) are also submitted.

**Table 2**: Bioinformatics results requested to be submitted for EURGen-RefLabCap EQA 2024.

| | **Bioinformatics results** |
|---|---|
| 1 | Prediction of the multi-locus sequence types |
| 2 | Detection of the plasmid replicon types |
| 3 | Detection of antimicrobial resistance genes |
| 4 | Detection of chromosomal point mutations mediating antimicrobial resistance |
| 5 | *In silico* prediction of antimicrobial resistance profiles |

**Important!!** In the 2024 EQA, both WS1 and WS2 pathogens are included. Please note that when reporting results for detection of ARGs/chromosomal PMs, only report genes and mutations which confer resistance to the relevant antimicrobials for each respective bacterial species. The lists of antimicrobials relevant for WS1 and WS2 pathogens for the current EQA are provided in Table 3 and Table 4, respectively. Please note that some antimicrobials listed for WS1 or WS2 might only be relevant for one of the species of that workstream. Please note that intrinsic genetic determinants are not part of the expected results. Please note that intrinsic AMR profiles (antimicrobials to which a particular species is intrinsically resistant) are not part of the expected results.

**Table 3.** Antimicrobial agents relevant for WS1 pathogens (*E. coli* and *K. pneumoniae*)

| Serial no. | Antimicrobial agent |
|---|---|
| 1 | Amikacin |
| 2 | Amoxicillin-clavulanic acid |
| 3 | Ampicillin |
| 4 | Aztreonam |
| 5 | Cefepime |
| 6 | Cefotaxime |
| 7 | Ceftazidime |
| 8 | Ceftazidime-avibactam |
| 9 | Ciprofloxacin |
| 10 | Colistin |
| 11 | Ertapenem |
| 12 | Fosfomycin |
| 13 | Gentamicin |
| 14 | Imipenem |
| 15 | Meropenem |
| 16 | Piperacillin-tazobactam |
| 17 | Sulfamethoxazole |
| 18 | Tigecycline |
| 19 | Tobramycin |
| 20 | Trimethoprim |

**Table 4.** Antimicrobials relevant for WS2 pathogens (*A. baumannii* and *P. aeruginosa*)

| Serial no. | Antimicrobial agent |
|---|---|
| 1 | Amikacin |
| 2 | Aztreonam |
| 3 | Cefepime |
| 4 | Ceftazidime |
| 5 | Ceftazidime-avibactam |
| 6 | Ciprofloxacin |
| 7 | Colistin |
| 8 | Fosfomycin |
| 9 | Gentamicin |
| 10 | Imipenem |
| 11 | Meropenem |
| 12 | Piperacillin-tazobactam |
| 13 | Tobramycin |

The use of the '*EURGen-RefLabCap harmonized common WGS-based genome analysis methods and standard protocols for national CCRE surveillance and integrated outbreak investigations*' and/or '*EURGen-RefLabCap harmonized common WGS-based genome analysis methods and standard protocols for national surveillance and integrated outbreak investigations of C/CRPa and C/CRAb'* (see https://www.eurgen-reflabcap.eu/resources/protocols-and-guidelines) is suggested, but the participants may choose to use their own WGS analysis set-up currently implemented at their laboratory. Thus, the participants are also asked to report method-related details in relation to the bioinformatics tools and parameters used for generation of results and sequence analyses, in addition to details of handling the received test materials and their sequencing (if performed).

# 4. Submission of results

In the EQA 2024, participants may decide to submit results for all the test material or may decide to submit data for some of the test materials. Participants may choose to submit all or any of the following;

i) raw sequencing data (FASTQ files) generated by the participating laboratories using live cultures (Item 1) using short-read OR long-read sequencing technology. The sequencing data uploaded by the participants will be evaluated for quality and results will be shared with the participating laboratories.

ii) raw sequencing data (FASTQ files) generated by the participating laboratories using pre-isolated DNA (Item 2) using short-read OR long-read sequencing technology. The sequencing data uploaded by the participants will be evaluated for quality and results will be shared with the participating laboratories.

iii) bioinformatics results via the EURGen-RefLabCap EQA 2024 webtool, referring to the test material codes listed in Table 1 and the analyses listed in Table 2.

## 4.1. Submission of information on methods for sequence analysis and test results

While proceeding with the analysis, participants are invited to register relevant information using the webtool. The questions in the webtool are presented in Appendix 4 (test forms). The laboratories must indicate the specifics of their procedures for each test material under the

"Method" section in the webtool. Here, for each test material, the laboratory will be asked to provide information about the type of test materials used and submitted results for i.e., if live cultures and/or pre-isolated DNA were used as test material, or if they used sequence data received from the EQA providers (short- or long-read sequence data, either in the FASTQ or FASTA format). In the "Method" sections participants should also indicate if the results for the specific test material are not submitted. See section 5 below for more details on the webtool.

### 4.2. Results related to antimicrobial resistance (AMR)

Acquired AMR genes (non-intrinsic) that contribute to the AMR profiles (mediating resistance to any of the antimicrobials included in this EQA for the respective bacterial species (Table 3 and 4)) should be included in the data submission. For reporting of ARGs, if the results show several genes and/or variants located in the same region on the contig or genome, only report the gene/variant which has the best quality in terms of percentage of coverage and identity. Please report the exact variant of the gene identified. Details for the submission of information on sequence analysis methods and sequence analysis results via the webtool are described in the webtool manual (Appendix 3).

Please note that the analysis might require collaboration between a bioinformatician and a microbiologist with knowledge within the field of AMR.

### 4.3. Results related to multi-locus sequence typing (MLST)

For MLST, you will be able to add allelic numbers for the seven housekeeping genes included in the MLST scheme. For *Acinetobacter baumannii* and *Escherichia coli*, there are several MLST schemes available. Kindly use the Oxford scheme for *A. baumannii* (*A. baumannii*#1, if using CGE MLST tool) and Achtman scheme for *E. coli* (*E. coli*#1, if using CGE MLST tool). Table 5 shows the seven alleles of the four bacterial species covered in the EQA 2024. If multiple alleles/sequence types (STs) with multiple perfect hits are detected, please report only the allele/ST which has the lowest number. For example, if you detect both allele 10 and allele 15 for *gyrB (E. coli*) with 100% identity and coverage, report allele 10. Similar guidelines apply while reporting ST.

**Table 5.** Alleles included in the multi-locus sequence typing (MLST) schemes relevant for EQA 2024

| Species | Allele 1 | Allele 2 | Allele 3 | Allele 4 | Allele 5 | Allele 6 | Allele 7 |
|---|---|---|---|---|---|---|---|
| *Escherichia coli (Achtman scheme)* | *adk* | *fumC* | *gyrB* | *icd* | *mdh* | *purA* | *recA* |
| *Klebsiella pneumoniae* | *gapA* | *infB* | *mdh* | *pgi* | *phoE* | *rpoB* | *tonB* |
| *Acinetobacter baumannii (Oxford scheme)* | *cpn60* | *gdhB* | *gltA* | *gpi* | *gyrB* | *recA* | *rpoD* |
| *Pseudomonas aeruginosa* | *acsA* | *aroE* | *guaA* | *mutL* | *nuoD* | *ppsA* | *trpE* |

## 4.4. Submission of raw sequencing data using ScienceData platform

For submission of raw sequencing data (FASTQ files), the ScienceData data platform will be used. Each participant is assigned a laboratory ID (Lab ID) and will be provided with a unique link to their individual folder on the ScienceData platform where they can upload the FASTQ files. Participants can find their Lab ID on the cover letter which is included as a hardcopy with the shipment of the test material, also forwarded as a pdf document via email to the contact persons.

Single-end or paired-end FASTQ files can be submitted for the evaluation. Before submission, rename FASTQ files to match with the corresponding test material. For example, paired-end FASTQ files generated from related to EURGen-2024-01_BACT should be named as EURGen-RLC-0XX_EURGEN-2024-01_BACT_R1.fastq.gz and EURGen-RLC-0XX_EURGEN-2024-01_BACT_R2.fastq.gz, while single-end FASTQ files should be named as EURGen-RLC-0XX_EURGEN-2024-01_BACT.fastq.gz (see also Table 6). In the file names, EURGen-RLC-0XX refers to the unique Lab ID that has been assigned to each participant (refer to the cover letter for the Lab ID).

**Table 6:** Templates of the file names to use while submitting sequences to EQA 2024 organizers for analyses

| Test strain ID | Material type | Paired/ Single-end | Raw data files names |
|---|---|---|---|
| **EURGEN-2024-01** | BACT | Paired | EURGen-RLC-**0XX**_EURGEN-2024-01_BACT_R1.fastq.gz<br>EURGen-RLC-**0XX**_EURGEN-2024-01_BACT_R2.fastq.gz |
| | | Single | EURGen-RLC-**0XX**_EURGEN-2024-01_BACT.fastq.gz |
| | DNA | Paired | EURGen-RLC-**0XX**_EURGEN-2024-01_DNA_R1.fastq.gz<br>EURGen-RLC-**0XX**_EURGEN-2024-01_DNA_R2.fastq.gz |
| | | Single | EURGen-RLC-**0XX**_EURGEN-2024-01_DNA.fastq.gz |
| **EURGEN-2024-02** | BACT | Paired | EURGen-RLC-**0XX**_EURGEN-2024-02_BACT_R1.fastq.gz<br>EURGen-RLC-**0XX**_EURGEN-2024-02_BACT_R2.fastq.gz |
| | | Single | EURGen-RLC-**0XX**_EURGEN-2024-02_BACT.fastq.gz |
| | DNA | Paired | EURGen-RLC-**0XX**_EURGEN-2024-02_DNA_R1.fastq.gz<br>EURGen-RLC-**0XX**_EURGEN-2024-02_DNA_R2.fastq.gz |
| | | Single | EURGen-RLC-**0XX**_EURGEN-2024-02_DNA.fastq.gz |
| **EURGEN-2024-03** | BACT | Paired | EURGen-RLC-**0XX**_EURGEN-2024-03_BACT_R1.fastq.gz<br>EURGen-RLC-**0XX**_EURGEN-2024-03_BACT_R2.fastq.gz |
| | | Single | EURGen-RLC-**0XX**_EURGEN-2024-03_BACT.fastq.gz |
| | DNA | Paired | EURGen-RLC-**0XX**_EURGEN-2024-03_DNA_R1.fastq.gz<br>EURGen-RLC-**0XX**_EURGEN-2024-03_DNA_R2.fastq.gz |
| | | Single | EURGen-RLC-**0XX**_EURGEN-2024-03_DNA.fastq.gz |

| Test strain ID | Material type | Paired/ Single-end | Raw data files names |
|---|---|---|---|
| **EURGEN-2024-04** | BACT | Paired | EURGen-RLC-**0XX**_EURGEN-2024-04_BACT_R1.fastq.gz<br>EURGen-RLC-**0XX**_EURGEN-2024-04_BACT_R2.fastq.gz |
| | | Single | EURGen-RLC-**0XX**_EURGEN-2024-04_BACT.fastq.gz |
| | DNA | Paired | EURGen-RLC-**0XX**_EURGEN-2024-04_DNA_R1.fastq.gz<br>EURGen-RLC-**0XX**_EURGEN-2024-04_DNA_R2.fastq.gz |
| | | Single | EURGen-RLC-**0XX**_EURGEN-2024-04_DNA.fastq.gz |

Upon uploading the files on ScienceData, please make sure that the size of the files corresponds to the expected file size. We also request the participants to upload the MD5 Checksum for each sequencing file they have uploaded to ScienceData. The MD5 Checksum is used to verify the integrity of files, as virtually any change to a file will cause its MD5 hash to change. For detailed information on how to upload files and MD5 Checksum onto ScienceData, please consult Appendix 2.

Files available in your ScienceData folder by the submission deadline will be considered for evaluation. Subsequently, pre-screening steps will be performed to confirm the sequence file format (FASTQ) and file name (as described above). If the file format and file names are not compatible with the submission guideline, the file will be excluded from further analysis.

### 4.5. Deadline for submission of results and sequencing data

Submission of test results is successful after ticking off the 'final submit' in the webtool (see webtool manual, Appendix 3). Following 'final submit', the laboratory contact person will receive an email with the submitted results as an attachment.

Results and the raw sequencing data must be submitted electronically **no later than 28 June 2024 at 16:00 CET**. Immediately after this, the webtool will be closed for further edits and submission. Delayed submission of results will not be accepted.

# 5. How to submit results via the webtool

The webtool manual (Appendix 3) presents the procedure of submission of information and results in detail. Please read it carefully before starting the results submission.

When the webtool is open for access, participants will receive their personal login ID and password via email. This is relevant for each email address provided when registering as participant in the EQA. Access the webtool using this URL and using Incognito mode in the browser: https://eurgen-reflabcap-pt.dtu.dk

When submitting results, kindly have the completed test forms (Appendix 4) with you. Before finally submitting results for the EURGen-RefLabCap EQA 2024, participants are asked to ensure that they have filled in all the relevant fields as **it is possible to 'finally submit' only once!** 'Final submit' blocks any further attempt of data entry.

**Do not hesitate to contact the EQA providers if you experience difficulties with the webtool.**

# 6. Evaluation

## 6.1. Evaluation and validation of the test results obtained from the analysis of the test material

The evaluation will be based on the test results submitted by the submission deadline based on the scoring regime presented in Appendix 3. Upon data validation, participants will receive an email message informing them that they may log in to the webtool once again to view and print an automatically generated individual evaluation report presenting their results. The webtool will allow the participants also to download a "certificate of participation" stating that the laboratory has participated in the specific EQA, without indicating pass/fail.

Evaluation criteria relevant to the submitted results are presented in the webtool manual (see Appendix 3). The reported details in relation to participants' analysis method will be used as background for the evaluation of results.

## 6.2. Evaluation of submitted raw sequences (FASTQ files)

The submitted raw sequence data (FASTQ files) will be evaluated using a quality control (QC) pipeline by the EQA 2024 organizers. The pipeline analyses the quality of sequences generated by short- and long-read technologies. The QC analysis will evaluate several parameters and depending on the sequencing technology used, the pipeline may include (but not limited to) parameters such as number of reads, number of contigs, coverage, N50, MLST type and Q-score.

When receiving the evaluation reports each participant is encouraged to assess their own performance and consider whether the obtained results should lead to adjustments of their DNA extraction, WGS and/or bioinformatics approaches.

## 6.3. Analysis and publication of results

Participating laboratories will receive an overall report summarizing the aggregated results of the EQA in an anonymized form (including likely explanations for discrepant results). The overall report will undergo written consultation with the EQA participants and will be shared with HaDEA and ECDC, and afterwards will be publicly available on the EURGen-RefLabCap website.

If relevant, the results of the EQAs will be published as scientific publications, also in an anonymized way, after receiving permission from all participating laboratories. In this case, authors and co-authors of the publications will be those who have contributed to the preparation and execution of the EQA. Due to the anonymity of performance results, the individual participating coordinators and colleagues in the laboratories will not be included as co-authors. Instead, the participating laboratories will be asked if they would like to be acknowledged in the publications, and by which specific laboratory name, place, and organization.

We thank you for your participation and we are looking forward to receiving your results.

**If you have any questions or concerns, please do not hesitate to contact us**.

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# Appendix 1

## Methods used to generate the EURGen-RefLabCap EQA 2024 test material (DNA, FASTA and FASTQ sequences)

**Table 1.** Methods used to extract DNA from test strains in EURGen-RefLabCap EQA 2024.

| | **Pre-isolated DNA** |
|---|---|
| **DNA extraction kit** | Invitrogen™ Easy-DNA™ gDNA Purification Kit |
| **DNA extraction protocol** | Invitrogen™ Easy-DNA™ Kit user guide |

**Table 2.** Methods used to generate FASTQ and FASTQ files corresponding to test strains in EURGen-RefLabCap EQA 2024.

| | **FASTA and FASTQ sequences generated using Illumina technology** | **FASTA and FASTQ sequences generated using Nanopore technology** |
|---|---|---|
| **Library preparation kit** | Nextera XT DNA Library Preparation Kit (96 samples) | Rapid sequencing DNA V14 - barcoding kit (SQK-RBK114.96) |
| **Library preparation protocol** | Illumina® Nextera XT DNA Library Prep Reference Guide (Version 06, August 2021). | Rapid sequencing DNA V14 - barcoding protocol (SQK-RBK114.96, VERSION: RBK_9176_V114_REVA_27NOV2022) |
| **Sequencing Platform used** | Illumina® NextSeq™ 500 | Oxford Nanopore GridION™ using R10.4.1 flowcells (FLO-MIN114). |
| **How QC of raw reads was performed** | FastQC v0.11.5 | NanoStat v1.4.0 |
| **How assembly of raw reads was performed** | Spades v3.15.3 | Flye v2.9.1 |

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# Appendix 2

## *Using ScienceData platform to transfer sequencing files*

ScienceData is a cloud-based data storage platform developed and operated by the Technical University of Denmark (DTU).

To obtain access to the platform, please use the link provided by the EQA organizers. The link will be sent via email to those who registered as contact persons via the sign-up form. For accessing ScienceData, it is recommended to use **Google Chrome** web browser.

In this appendix, you will find instructions regarding;

- Uploading of raw sequencing data (FASTQ files) onto ScienceData platform for Quality control (QC)
- Downloading of sequencing data (FASTA and FASTQ files) from ScienceData

## Uploading files to the ScienceData platform

Obtain access to the platform by using the link provided to you in the cover letter, included as a hardcopy with the shipment of the PT material, and also forwarded as a pdf document by email to the EQA contact persons. Please refer to the EQA 2024 protocol for templates for naming sequencing data files (Table 6).

1. Click on the link provided by the EQA organizer and you will see the following page (example):


*Image 1. An example of upload page on ScienceData platform*

2. Click on the “Upload” button to choose and upload your files.

3. Confirm that the sizes of the transferred files on ScienceData correspond to the expected file sizes.

4. We encourage the participants to upload the MD5 Checksum for each sequencing file they have uploaded to ScienceData. Most commonly, md5sum is used to verify that a file has not changed because of a faulty file transfer, a disk error or non-malicious modification. To upload MD5 Checksum, compile the hash values corresponding to the FASTQ files in a text file as shown in the snapshot below (Image 2). Please name the file as EURGen-2024-**0XX**.txt (0XX refers to the unique Lab ID that has been assigned to each participant (see the cover letter for the Lab ID))

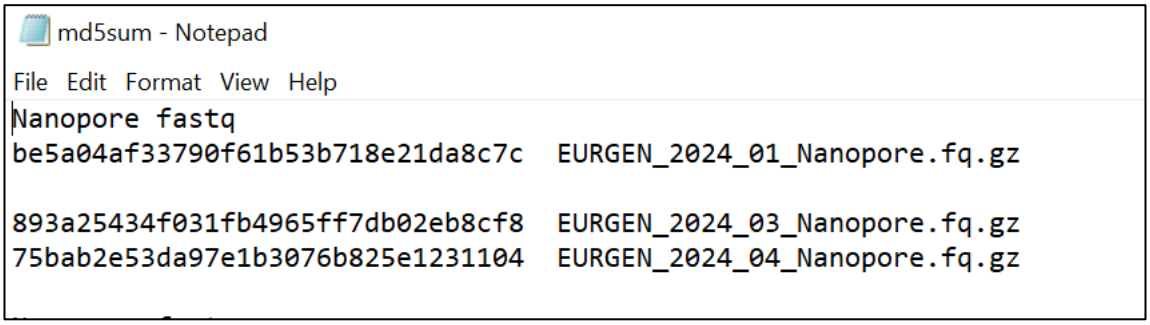



*Image 2. An example of text file containing MD5 Checksum hash values for fastq files*

## Downloading files from the ScienceData platform:

1. Click on the link provided by the EQA organizer and you will reach the ScienceData platform where you will see two folders, “FASTA” and “FASTQ”. See image 3.


*Image 3. FASTA and FASTQ folders on ScienceData platform*

2. In the “FASTA” folder, you will see four two subfolders, “FASTA_Illumina” and “FASTA_Nanopore”. Click on the folder to choose the files you wish to download. You should see the following sequences:

a) Subfolder FASTA_Illumina (4 files)

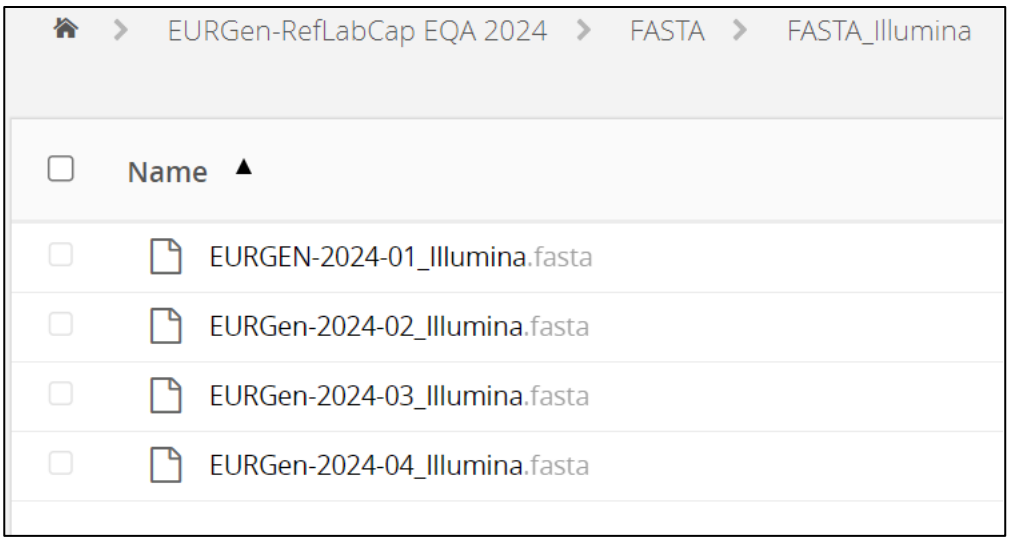



*Image 4. FASTA files located in FASTA_Illumina subfolder*

b) Subfolder FASTA_Nanopore (4 files)

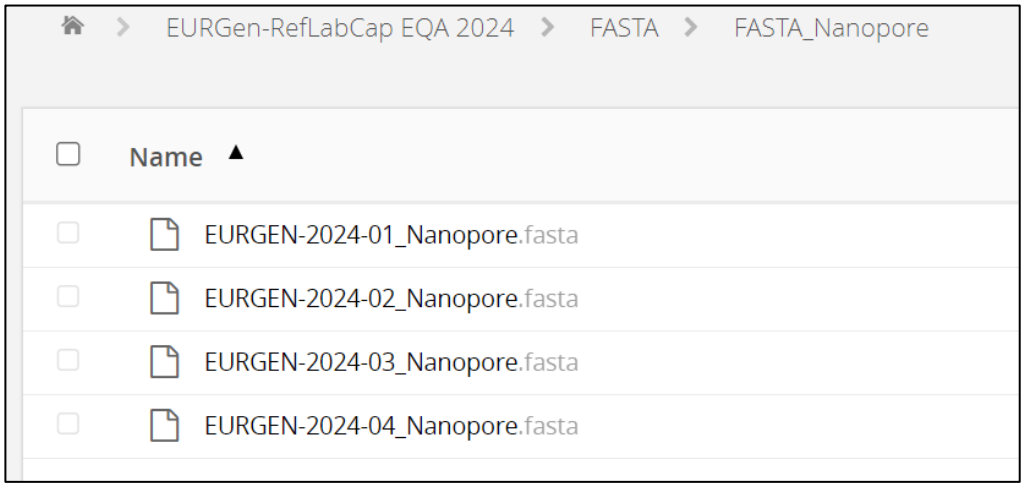



*Image 5. FASTA files located in FASTA_Nanopore subfolder*

3. In the “FASTQ” folder, you will see four two subfolders, “FASTQ_Illumina” and “FASTQ_Nanopore”. Click on the folder to choose the files you wish to download. You should see the following sequences:

a) Subfolder FASTQ_Illumina (8 files)

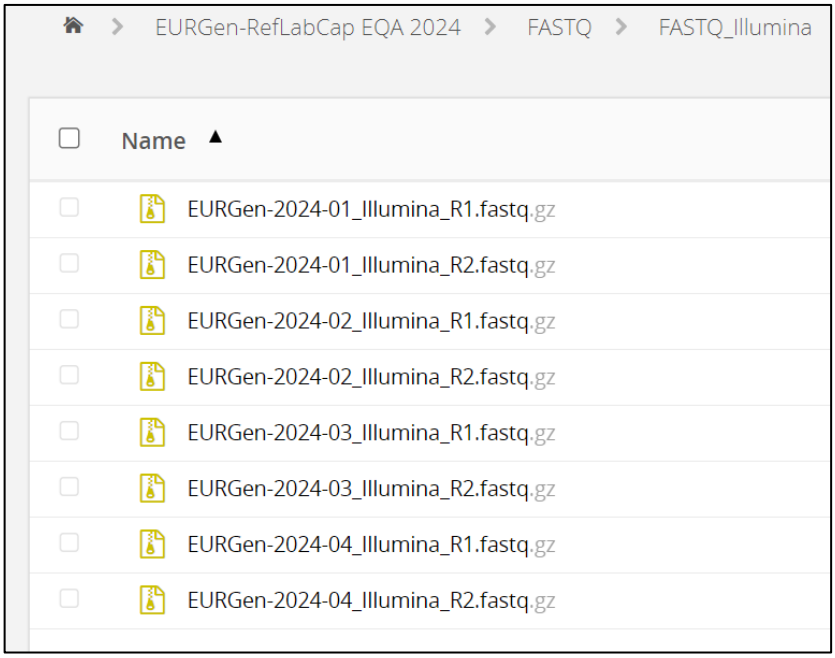



*Image 6. FASTQ files located in FASTQ_Illumina subfolder*

b) Subfolder FASTQ_Nanopore (4 files)

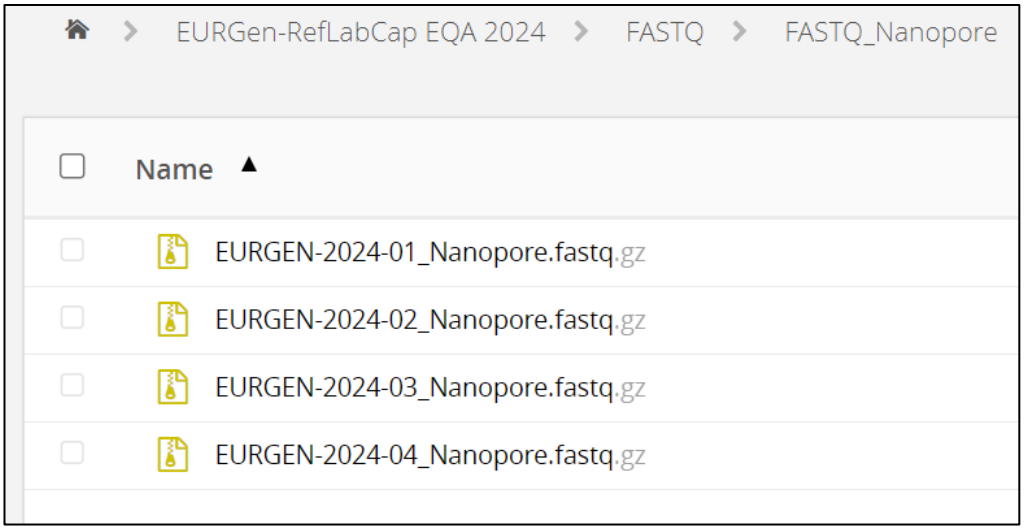



*Image 7. FASTQ files located in FASTQ Nanopore subfolder*

4. To download, select the file(s) you want to download and click the “download” button as shown in Image 8.

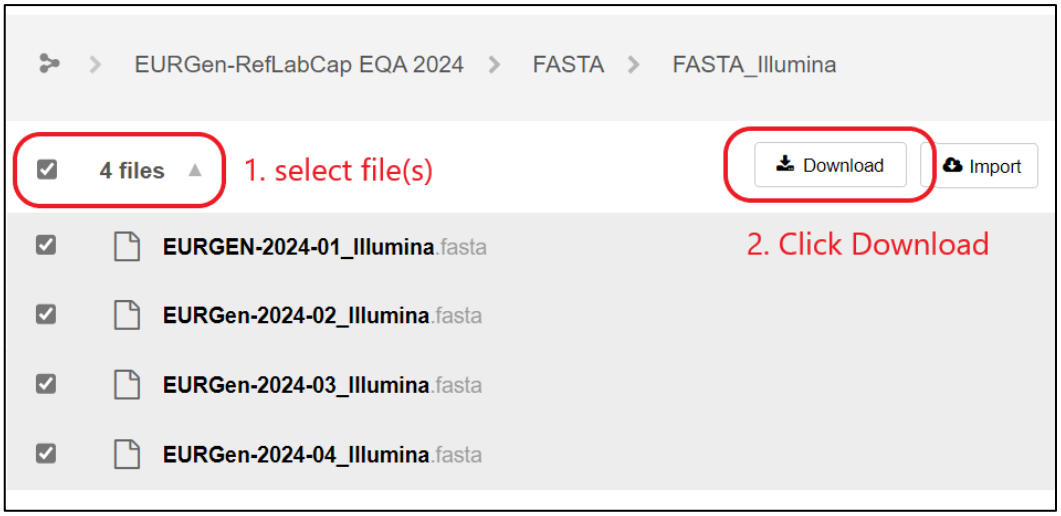



*Figure 8. Downloading files from ScienceData*

--- --- ---

# Appendix 3

## *EURGen-RefLabCap EQA 2024 webtool manual*

## Browser requirements

**IMPORTANT:** The system works with the following browsers

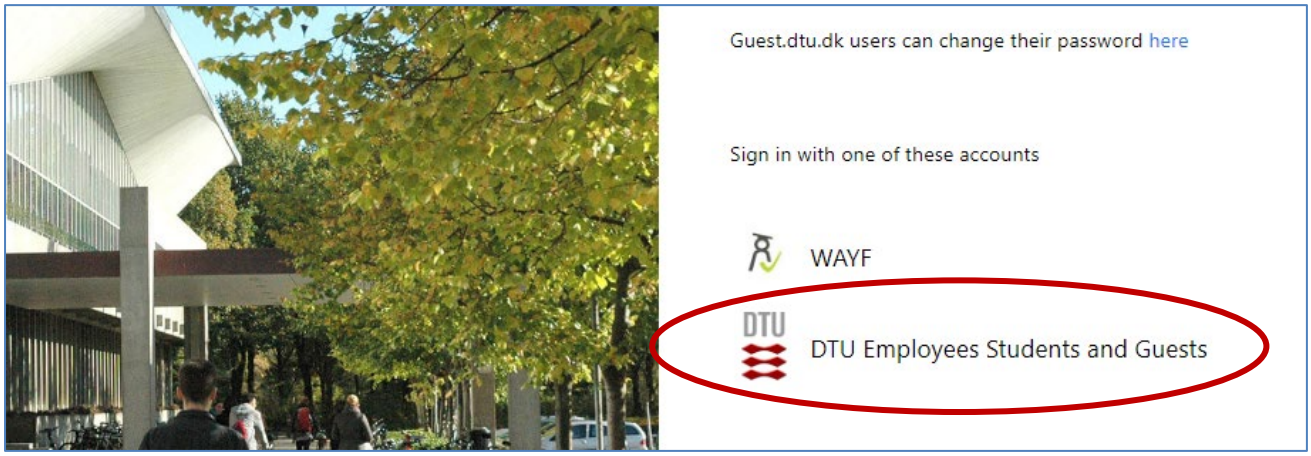
| Browser | Oldest supported version* |
|---|---|
| Google Chrome | 44.0 |
| Firefox | 39.0 |

* latest version is recommended

## Accessing the webtool

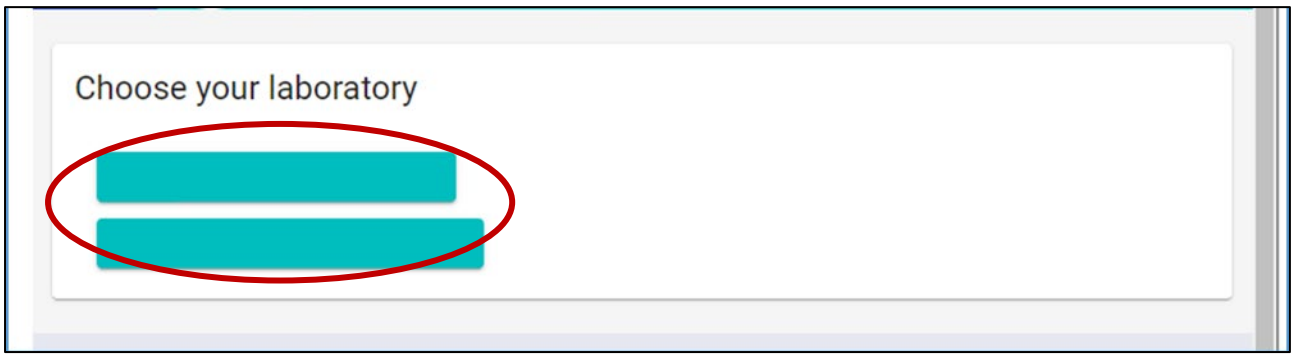
Please find information for support and suggestions for **troubleshooting** below in this document.

Access the webtool *Sign-in* page via this link: https://eurgen-reflabcap-pt.dtu.dk




When reaching the image above by clicking the webtool link, click “DTU Employees Students and Guests”.

Login to the webtool by using the username and password sent to you by e-mail for participation in EQAs. After signing in, you will reach the *Overview* page.



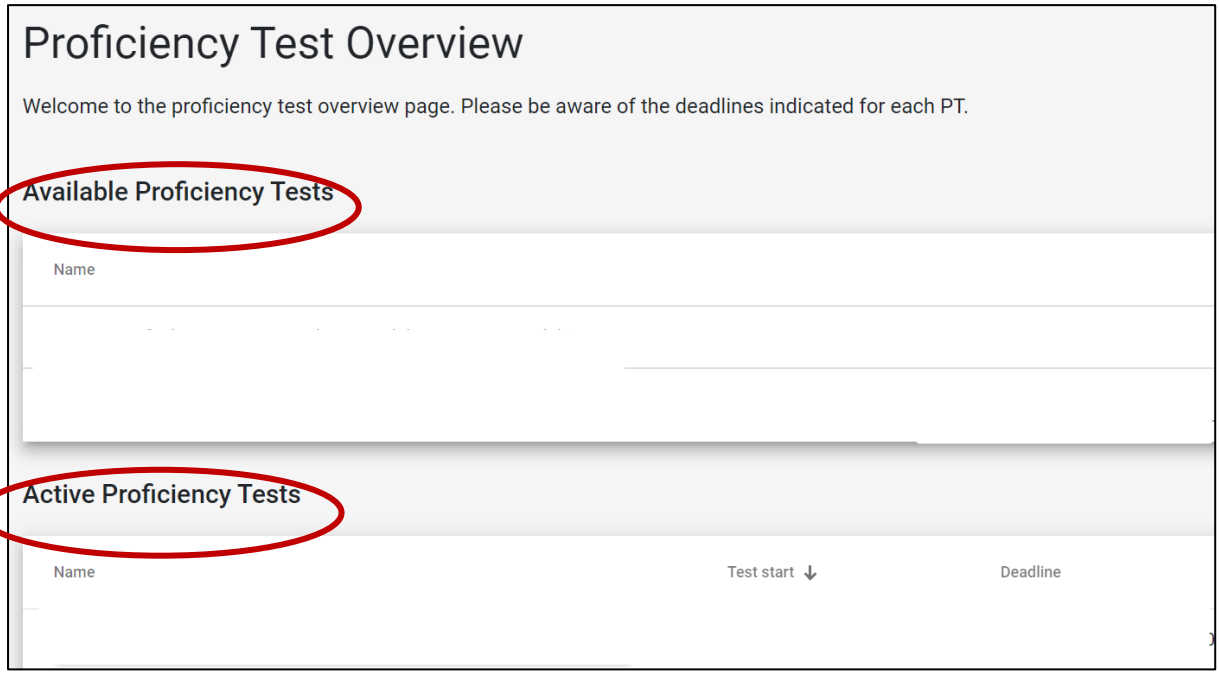

If you are connected to more than one specific laboratory, you will need to select the specific laboratory that you intend to submit results for.

If the window has been inactive for 20 minutes, the webtool will automatically time-out and present 'Access denied'. Access the webtool once again by following the above-described login procedure.

## Signup or deselect

For EQA 2024, individual results submission webtools are available for submission of results, i.e., laboratories that signed up for analysing live bacterial cultures and/or DNA are invited to submit their results in EURGen-RefLabCap_EQA2024_BACT-DNA, while laboratories which requested FASTA/FASTQ files corresponding to the test strains are invited to submit their results in EURGen-RefLabCap_EQA2024_FASTA-FASTQ. Please note that both webtools have the same layout and content, except for the questions under "Methods" tab. For detailed information about "Method" section, please refer to the appendix 4 (test forms).

Under "Available Proficiency Tests", sign up to the "EURGen-RefLabCap_EQA2024_BACT-DNA" or "EURGen-RefLabCap_EQA2024_FASTA-FASTQ". The EQA that you have signed up for, will be listed under 'Active Proficiency Tests'. Note for the current webtool, please read 'EQA' when reference to 'proficiency test' is made.




## Navigate in the webtool

When reporting results/data in the webtool, various tabs are available:

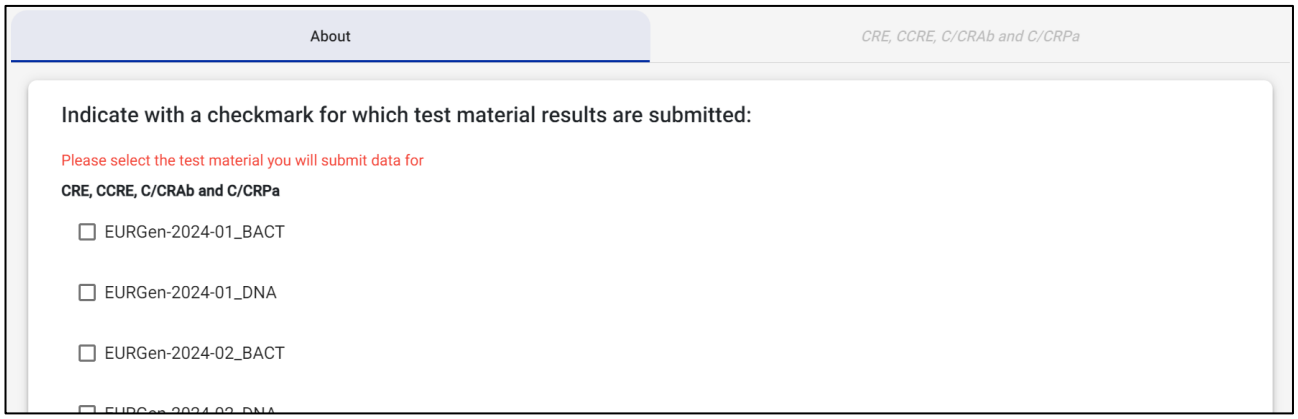
1. **'About' tab**. With a checkmark, select the test material for which you wish to submit results, i.e., select each relevant codes
2. Under the **'CRE, CCRE, C/CRAb and C/CRPa' tab**, find the tab:
    - **'Method':** Enter information regarding the sequencing and bioinformatics analyses performed
3. Under the **tab presenting each test material code**, find the tabs:
    - **'AMR':** Enter results regarding antimicrobial resistance genes, chromosomal mutations mediating antimicrobial resistance and predicted antimicrobial resistance profile
    - **'MLST':** Enter data regarding sequence type and alleles
    - **'Plasmid replicon':** Enter data regarding plasmid replicon type

## Enter data

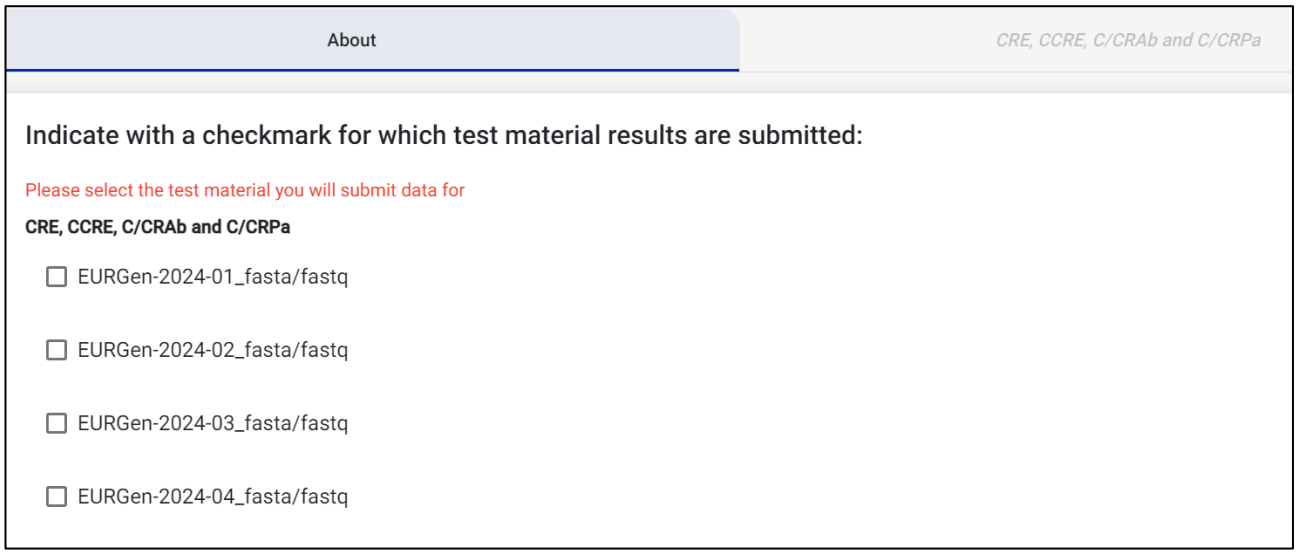
**Ad 1. 'About' tab**

In the "About" tab, select the test material that you want to submit results for. If a test material is

selected under the ‘About’ tab, remaining tabs related to the selected test material will be activated under “CRE, CCRE, C/CRAb and C/CRPa” tab.




*”About” tab in EURGen-RefLabCap_EQA2024_BACT-DNA*

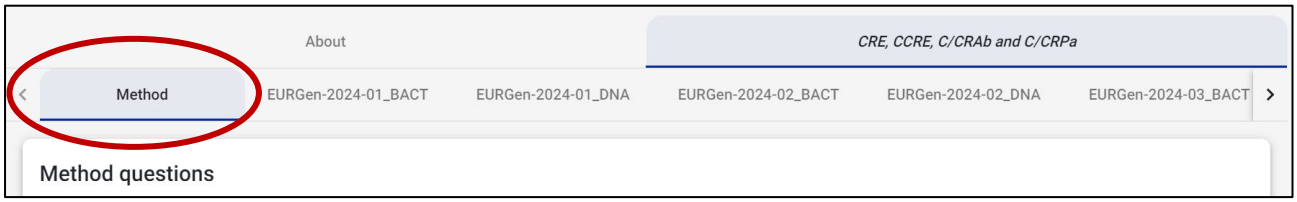



*”About” tab in EURGen-RefLabCap_EQA2024_FASTA-FASTQ*

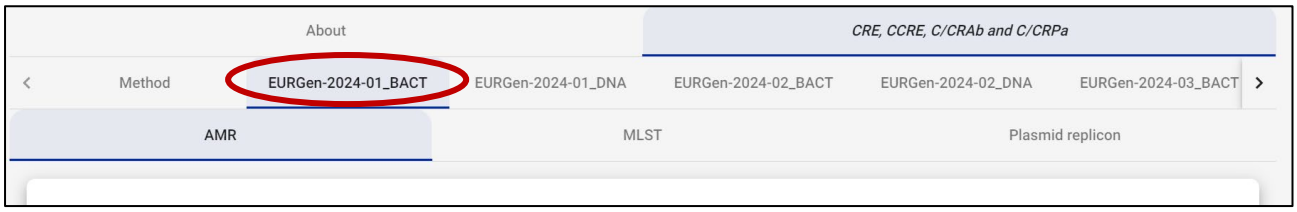
From here onwards, the screenshots are shown for “EURGen-RefLabCap_EQA2024_BACT-DNA”, only. The layout and content in EURGen-RefLabCap_EQA2024_FASTA-FASTQ is similar to EURGen-RefLabCap_EQA2024_BACT-DNA.

## Ad 2. 'Method' tab

Respond to the questions in the 'Method' tab.



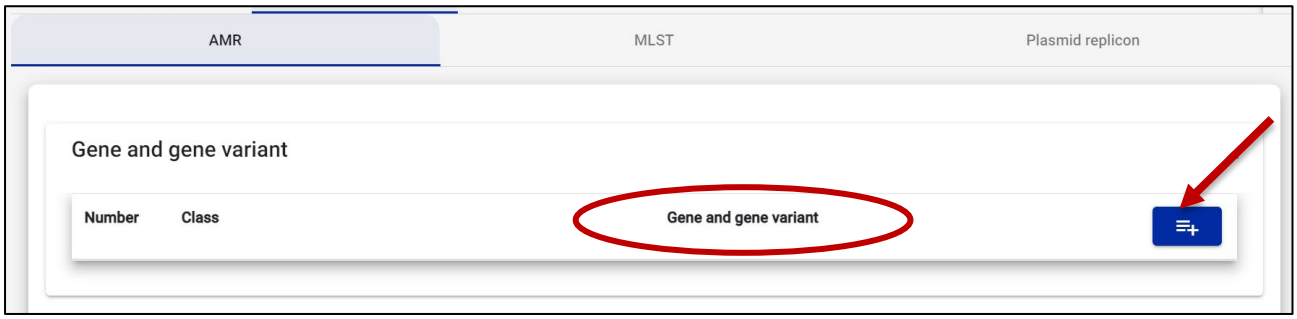

Select the tab related to one of the test materials (e.g., EURGen-2024-01_BACT). This opens access to additional tabs i.e., "AMR", "MLST", and "Plasmid replicon".



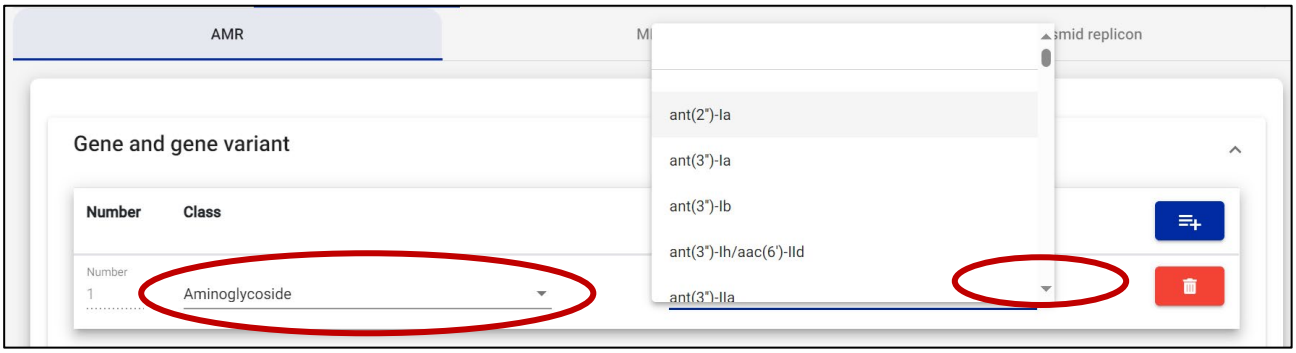

## Ad 3. 'AMR' tab:

Under the AMR-tab, results related to 1) identified antimicrobial resistance (AMR) genes, 2) identified chromosomal mutations mediating AMR, 3) and predicted of the AMR profiles are uploaded.

**Gene and gene variant**: To report genes and gene variants detected in the sequences of the test strains, please click on the '+' (see arrow below) to access the dropdown lists.




In the dropdown menu under 'Class', select the antimicrobial class of the gene and gene variant you wish to report. Hereafter, from the dropdown menu under 'Gene and gene variant', select the specific variant of the AMR gene you wish to report, or, to narrow down the options in the list, type (parts of) the gene variant name in the 'Filter'-field. To submit more genes, add more lines by clicking on the '+' button.



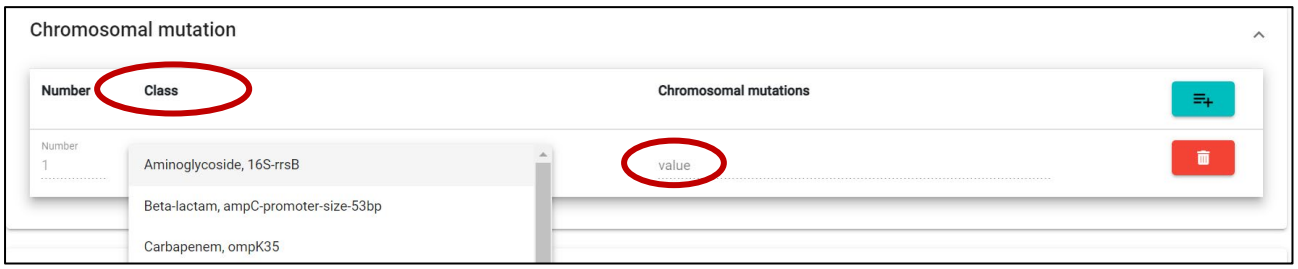

**Note**: AMR towards a limited number of antimicrobial classes is considered in this EQA. Antimicrobial classes represented in the EURGen-RefLabCap EQA 2024, and consequently in the webtool drop down list are presented in Table 3 and Table 4 of EQA 2024 protocol.

Some AMR genes are associated with resistance to more than one class of antimicrobials. To select these genes, select a 'Class' containing multiple classes and subsequently select the specific gene and gene variant.

Ensure that no empty lines are saved for evaluation by clicking on the bin if you by mistake added one too many.

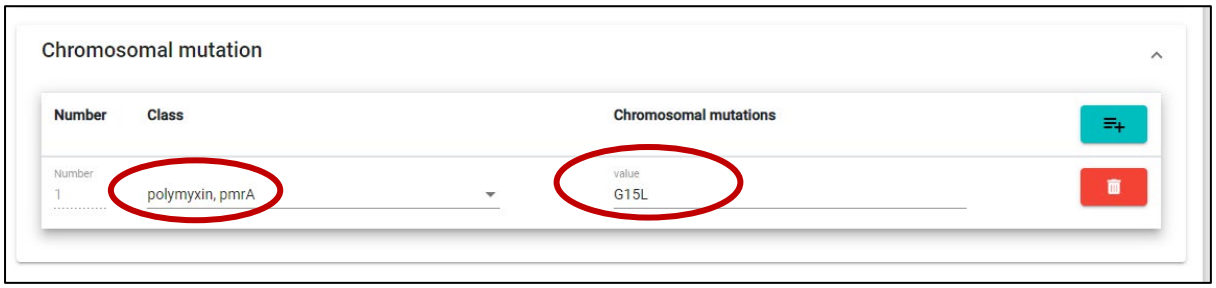

**'Chromosomal mutations':** In the dropdown menu under 'Class', select the specific class of antimicrobial followed by the gene for which you want to report a mutation. Hereafter, under 'Chromosomal mutations', in the empty field named "value", write the specific mutation as follows:

1) Indicate the reference codon (an amino acid letter(s), or a nucleotide(s) letter for 16S or 23S sequences)
2) Indicate the position of the codon (a numeric value)
3) Indicate the resistance codon (amino acid letter(s), or nucleotide(s) for 16S or 23S sequences)

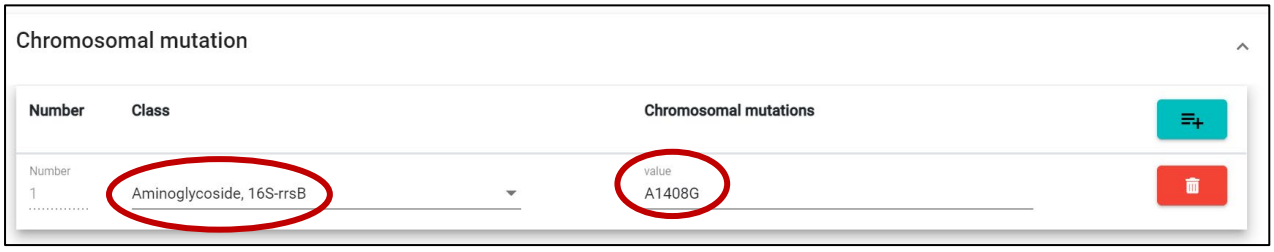



Please note that "value" field is a free text field without any restrictions on what can be typed in the field. Please follow the guidelines described above for adding single nucleotide polymorphism (SNP) and insertion mutations. See examples of different mutations below;

**Example 1:** Reporting a mutation in the *pmr*A gene which has changed the *amino acid* glycine (G) to Leucine (L) at position 15. This results in resistance to colistin that belongs to the polymyxin class of antimicrobials. Therefore, from the dropdown list under 'Class', select the 'polymyxin, *pmr*A' option and write G15L in the 'value' field under 'Chromosomal mutations'.

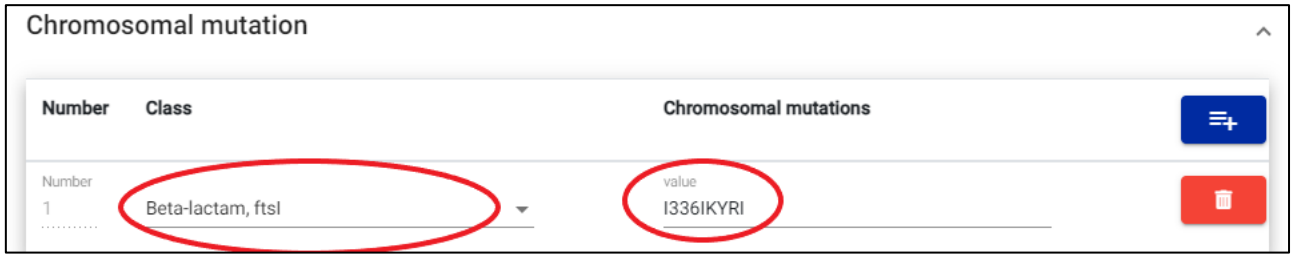



**Example 2:** If the mutation is in a 16S rRNA gene please select the class of antimicrobial and associate gene (e.g., Aminoglycoside, 16S-rrsB) from the dropdown menu. Hereafter, in the 'value', write the letter of the original reference *nucleotide* (A, T, C or G) and its position in the gene sequence, followed by the new nucleotide letter that the mutation has resulted in (e.g., A1408G). Same principle as for the amino acids.



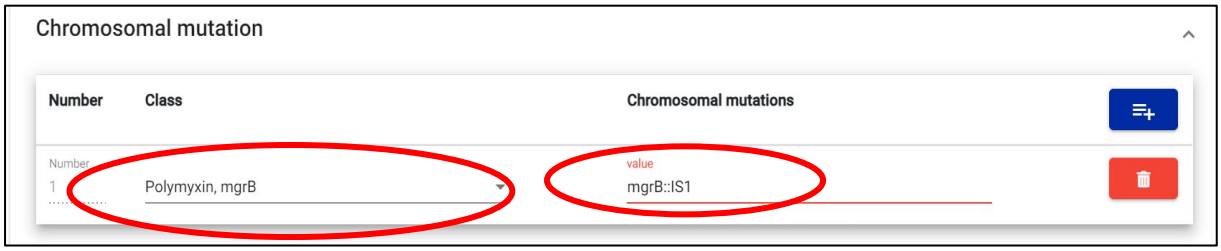

In the above example, the original nucleotide is "A", position in the gene sequence is "1408", and the new nucleotide that replaced the original nucleotide is "G".

**Example 3:** If you want to report a mutation which results in the insertion of multiple amino acids in a protein sequence (e.g., Beta-lactam, *ftsI*_I336IKYRI), select "Beta-lactam, *ftsI*" from the dropdown list under "Class" and write "I336IKYRI" in the "value filed under "chromosomal mutation", as highlighted in the image below".

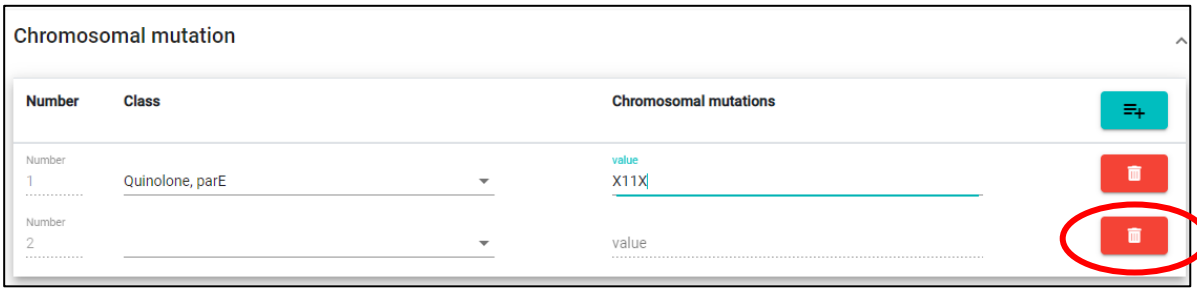



In the above example, 5 amino acids (IKYRI) are added after amino acid "I" at position "336" in *ftsI* protein.

**"Gene disruptions caused by IS elements"**: To add mutations caused by disruption of gene sequences (or in the promoter region) due to insertion sequence (IS) elements, select the gene from the drop down list under "class" and write the mutation as "gene affected::IS family". **For example**, to write mutation in *mgrB* caused by IS*1*, write *mgrB::*IS*1* in the value under "Chromosomal Mutations", as shown in the image below.



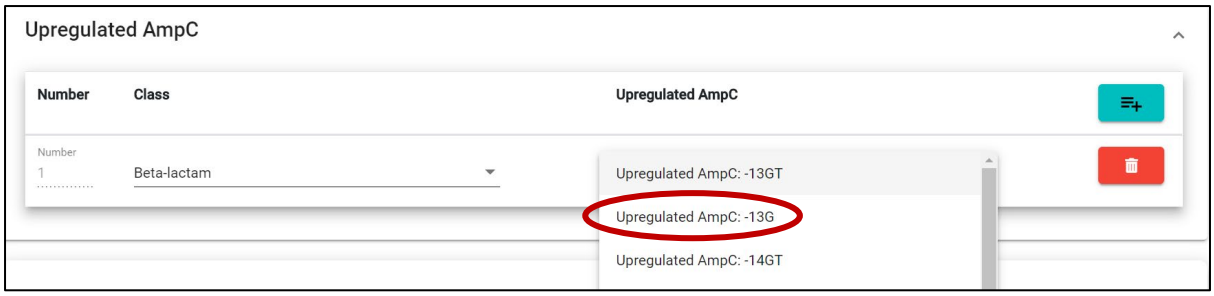

**Important!** Please make sure that no empty lines are saved for evaluation. If you have an empty entry, delete the entry by clicking on the "bin" button as shown in the image below.



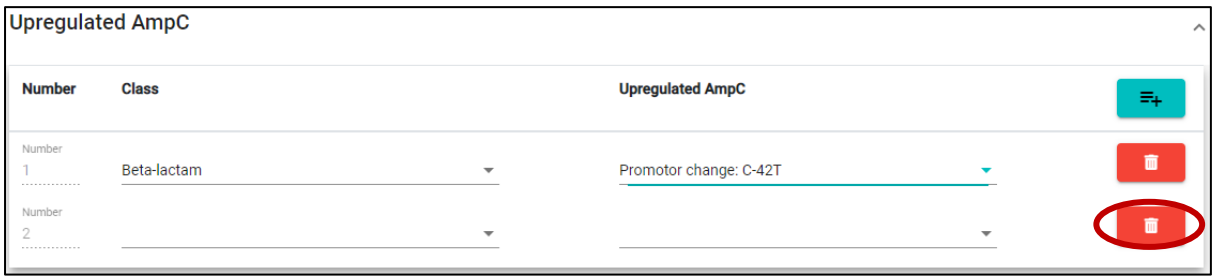

**Important**! Please double check the mutations that you have added and make sure that the mutations follow the nomenclature described here and there are no typing mistakes.

**'Upregulated ampC'**: Upregulated ampC resistance can be reported by selecting the 'Upregulated ampC' option under the 'AMR' tab. For the 'Upregulated ampC' option, select 'Beta-lactam' under 'Class' and hereafter, from the dropdown menu under 'Upregulated ampC', select the specific mutations in the promoter region.

The mutations are shown in the same way as previously described for 16S and 23S sequence mutations, i.e., the reference codon is followed by a numeric value, and then followed by the resistance codon (unless the mutation is an insertion). Since the promoter is located upstream to the open reading frame (ORF) a minus (-) is found before the position number. e.g., C-42T (indicating that the nucleotide cytosine (C) has been exchanged with thymine (T)). Regarding insertions, there is no reference nucleotide, therefore, for example, the indication '-13G' represents the nucleotide guanine (G) inserted at position -13 (upstream the ORF).



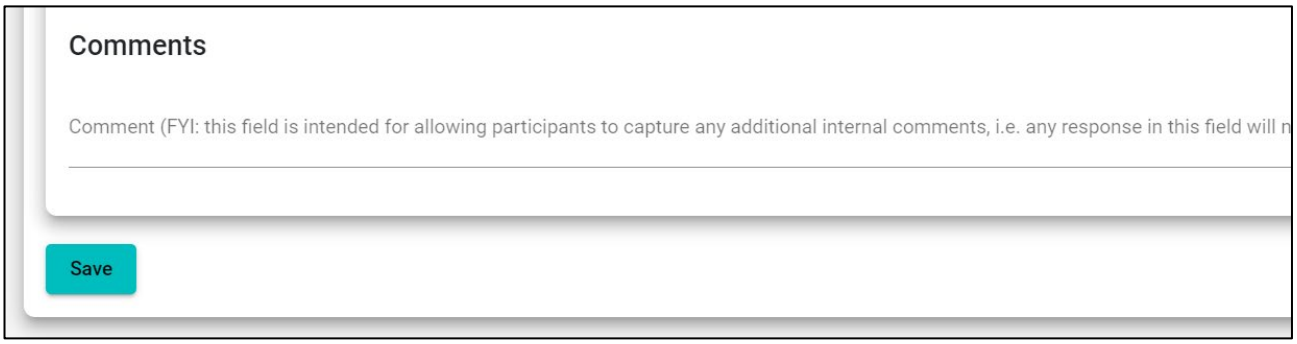

**Important!** Please make sure that no empty lines are saved for evaluation. If you have an empty entry, delete the entry by clicking on the "bin" button as shown in the image below.




**'Predicted AMR profile":** Click on 'Predicted AMR profile" to select the antimicrobial agents that test strain is predicted to be resistant to, based on the evaluation of the sequencing data. Note that in relation to predicted AMR profile, AMR towards a limited number of antimicrobials is considered in this EQA. Antimicrobials represented in the EURGen-RefLabCap EQA 2024 relevant for workstream 1 and workstream 2 pathogens are listed in Table 3 and Table 4 of the EQA 2024 protocol, respectively.


**'Comments':** Any comments related to the submission of the results are welcome. You may for example indicate mutations that have unknown effects on AMR. You may also report genes and/or mutations that were not listed in the drop-down menu of the tabs described above. Note, however, that these results will *not* be further evaluated.

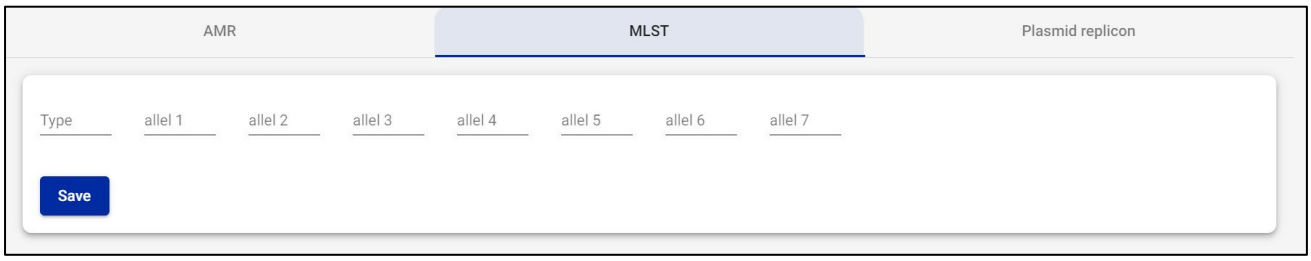
Click "Save" once all data in the "AMR" has been reported.




**Ad 4. 'MLST' tab**

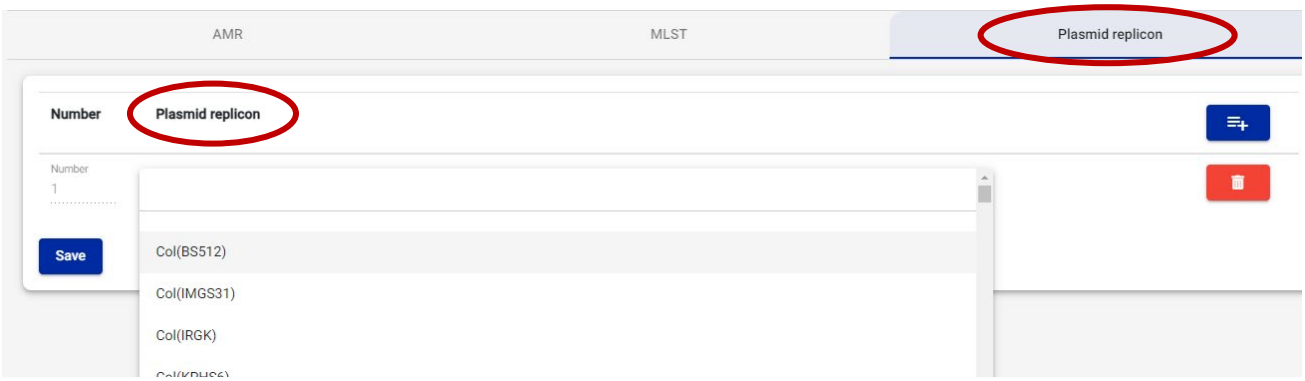
Enter data regarding multi-locus sequence typing (MLST) and corresponding allele numbers. Enter "0" if the obtained result does not show a perfect match or if an allele cannot be detected. Please

refer to the EQA 2024 protocol (section 4.3) for information regarding MLST schemes and alleles relevant for the EURGen-RefLabCap EQA 2024.




**Ad 5. 'Replicon' tab**

Enter data regarding plasmid replicon type by selecting from the dropdown list. If multiple copies of same plasmid replicon are detected, report it only once.




## Save data

Data is saved when you click the *save* button on each page. Moreover, data is saved when you navigate to another tab.

## Review and revise data

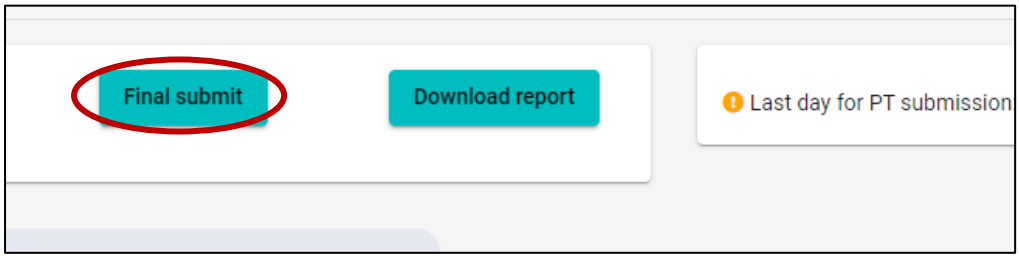
On the *Proficiency Test Overview* page as well as in the *Test overview page*, click 'Download report' to see the overview of your results and method input for this EQA.

Before you have finally submitted your results (and before deadline), the database allows you to return to any test form where you can make changes to the reported data.

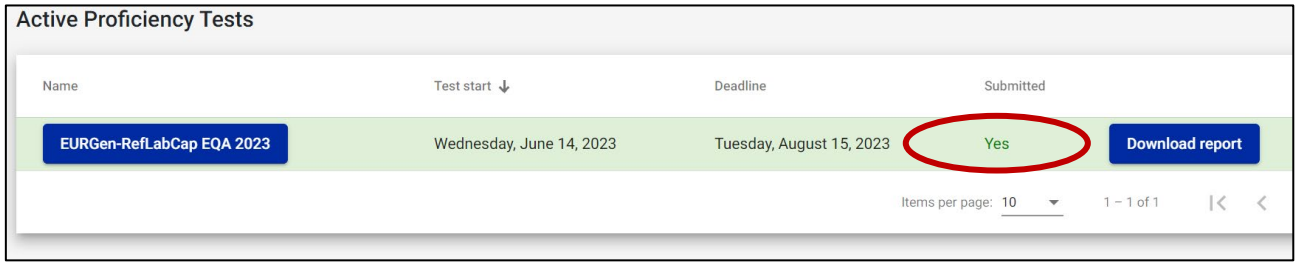
## Submit data

For all test materials of the EURGen-RefLabCap EQA 2024, all uploaded data are submitted in one go.

When all information and data have been entered and revised by the participants, click on the "Final submit" to submit your data. Please note that you will **NOT** be able to edit your data after final submission.




When you have finally submitted, the *Proficiency Test Overview* page will indicate the submission status of your Proficiency Test to be 'Yes'




## Evaluation and score

Submitted results are evaluated according to the following details:

The reported MLST will be compared to the MLST of the reference sequence. A match of the obtained and expected MLST is scored with '1'. A mismatch is scored with '0'.

Similarly, reported plasmid replicon type will be compared to the expected plasmid replicon type. Each reported replicon will be scored individually. When a submitted replicon is on the list of expected replicons (i.e., if the obtained and expected results match), a score of '1' will be achieved, whereas a mismatch (obtained result is not expected) is scored with '0'. If the submitted replicon is

expected but is non-mandatory to report will be scored as “blank”. Expected replicon which is not submitted as obtained results will be presented in the evaluation report and for these the score field will be blank.

Reported AMR genes will be compared to the expected results. Each reported gene will be scored individually. When a submitted AMR gene is on the list of expected AMR genes (i.e., if the obtained and expected AMR genes match), a score of ‘1’ will be achieved, whereas a mismatch (obtained result is not expected) is scored with ‘0’. If reported AMR gene is expected but not mandatory to report, it will be scored as “blank”. Expected AMR which are not submitted as obtained results will be listed in the evaluation report and for these the score field(s) will be blank.

The reported chromosomal mutations mediating AMR will be compared to the expected results. Each reported mutation will be scored individually. When a submitted chromosomal mutation is on the list of expected chromosomal mutations (i.e., if the obtained and expected results match), a score of ‘1’ will be achieved, whereas a mismatch (obtained result is not expected) is scored with ‘0’. If reported mutation is expected but not mandatory to report, it will be scored as “blank”. Expected chromosomal mutations which are not submitted as obtained results will be presented in the evaluation report and for these the score field will be blank.

Reported predicted AMR profile will be compared to the expected results. In the EQA 2024, each reported antimicrobial agent will be scored individually. If the obtained and expected results match, a score of ‘1’ will be achieved, whereas a mismatch is scored with ‘0’. If reported antimicrobial agent is expected but not mandatory to report, it will be scored as “blank”. Expected antimicrobial agents which are not submitted as obtained results will be presented in the evaluation report and for these the score field will be blank.

When the score is released and the evaluation reports are accessible, all participating laboratories will receive an email from the EQA organizer. Upon login to the database, clicking on ‘*Download report*’ will give access to the report presenting obtained results, expected results and scores.

The evaluation will not indicate pass/fail.

## Support

Should you need support in using the webtool, please do not hesitate to contact the EQA Coordinator (suska@food.dtu.dk).

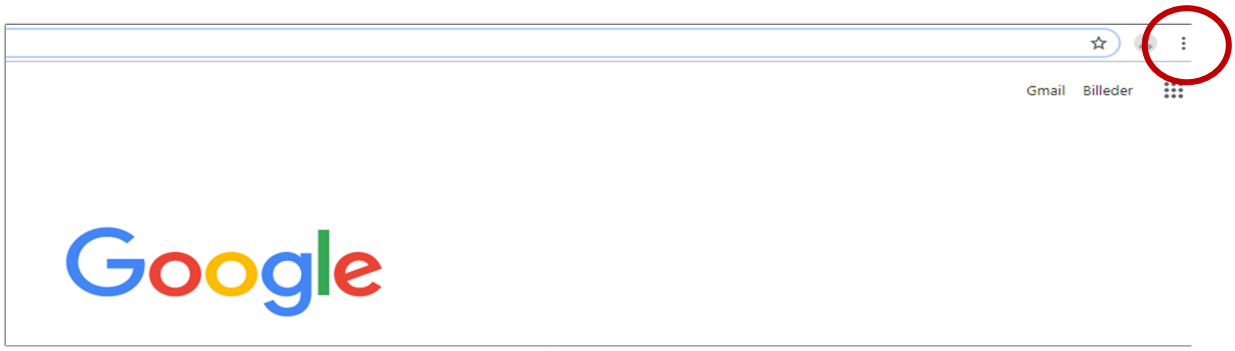
In top right corner of each page in the webtool, also find the name and email address for the EQA organizer including the link to the EURGen-RefLabCap website (to access the relevant EQA protocol) as well as access to the present webtool manual.

**TROUBLESHOOTING**: Should you encounter issues when attempting to access or upload results to the results submission webtool, it may be solved by one of the following.

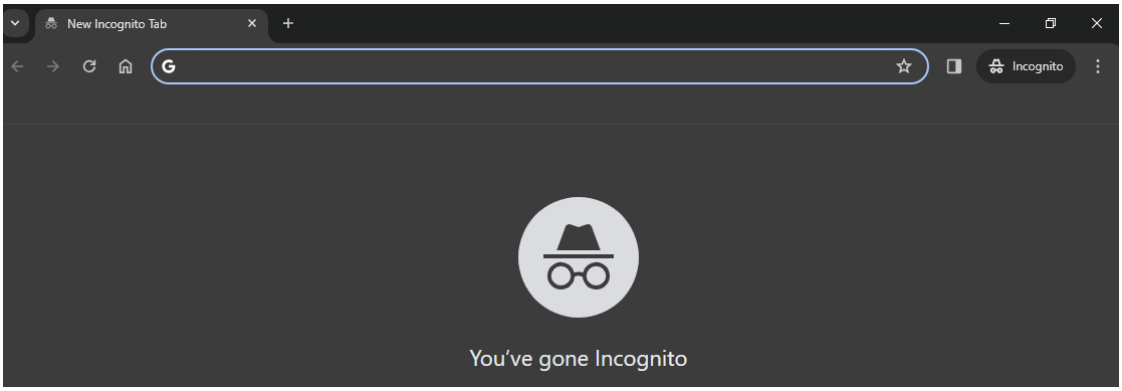
Please try to:

- Restart your browser
- Try using an incognito window (see instructions below)
- Instead of Wifi, use a cable Internet connection
- Use a connecting outside hospital/university firewalls – e.g., a private Internet connection

Try accessing the using an incognito window by opening a browser window, click on three dots (see red circle in the image below) and select: 'New incognito window' (relevant when using Google chrome).




You should see a dark incognito window similar to the image below (when using Google chrome):



Access the webtool by copying and pasting the following webtool-link to the address bar in the incognito window and press **Enter** on the keyboard. You will reach the webtool *Sign-in* page. Follow the instructions in the paragraph 'Accessing the webtool' at the top of this document.

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# Appendix 4

## *Test forms – overview of method information to be submitted via the webtool*

In this document, we present an overview of the data that may be submitted to the webtool.

You will find questions regarding:

- Test material used for EURGen-RefLabCap EQA 2024
- Storage and handling of live cultures (only relevant if you have used the live cultures as test material)
- Storage and handling of received DNA (only relevant if you have used DNA as test material)
- Sequencing method (only relevant if you have performed sequencing using the test material (live cultures and/or pre-isolated DNA)
- Quality control of sequence data (only relevant if you have performed sequencing using the test material (live cultures and/or pre-isolated DNA)
- Analyses of sequencing data: multi-locus sequence types, plasmid replicons, antimicrobial resistance (AMR) genes and chromosomal point mutations mediating AMR, *in silico* prediction of AMR profiles
- Sequencing data uploaded by EQA participants for quality control

In the EURGen-RefLabCap EQA 2024, laboratories are provided with the live bacterial cultures and pre-isolated DNA as test material. If, for some reason, the laboratory cannot sequence using the live cultures and pre-isolated DNA, FASTA or FASTQ files corresponding to the test isolates can be provided as test material. The results submission webtool captures data related to analysis of the live cultures and pre-isolated DNA or, in a separate webtool, related to the FASTA or FASTQ files. For both webtools, the laboratories must indicate the type of test material they have used for the analyses (live cultures and pre-isolated DNA, or sequence data (FASTQ or FASTA files produced through short-read or long-read sequencing)), or if results for the specific test material is not submitted. The webtool that captures results from analysis of FASTA or FASTQ files does not present method questions related to the handling of live cultures and pre-isolated DNA contains but is limited to the relevant questions of those presented below.

## Questions regarding the test material used for EURGen-RefLabCap EQA 2024

<u>In the webtool that captures data related to the analysis of live cultures and pre-isolated DNA</u>

1.1. Which type of the test material did you use for isolate EURGen-2024-01
   i) Both Live cultures and pre-isolated DNA
   ii) Only Live cultures

iii) Only pre-isolated DNA
iv) Not applicable

1.2. Which type of the test material did you use for isolate EURGen-2024-02
i) Both Live cultures and pre-isolated DNA
ii) Only Live cultures
iii) Only pre-isolated DNA
iv) Not applicable

1.3. Which type of the test material did you use for isolate EURGen-2024-03
i) Both Live cultures and pre-isolated DNA
ii) Only Live cultures
iii) Only pre-isolated DNA
iv) Not applicable

1.4. Which type of the test material did you use for isolate EURGen-2024-04
i) Both Live cultures and pre-isolated DNA
ii) Only Live cultures
iii) Only pre-isolated DNA
iv) Not applicable

<u>In the webtool that captures data related to the analysis FASTA-FASTQ files provided by EQA organizers</u>

1.1. Which type of the test material did you use for isolate EURGen-2024-01
i) FASTQ_Illumina files provided by EQA organizers
ii) FASTQ_Nanopore file provided by EQA organizers
iii) FASTA_Illumina file provided by EQA organizers
iv) FASTA_Nanopore file provided by EQA organizers
v) Not applicable

1.2. Which type of the test material did you use for isolate EURGen-2024-02
i) FASTQ_Illumina files provided by EQA organizers
ii) FASTQ_Nanopore files provided by EQA organizers
iii) FASTA_Illumina files provided by EQA organizers
iv) FASTA_Nanopore files provided by EQA organizers
v) Not applicable

1.3. Which type of the test material did you use for isolate EURGen-2024-03
i) FASTQ_Illumina files provided by EQA organizers
ii) FASTQ_Nanopore files provided by EQA organizers

iii) FASTA_Illumina files provided by EQA organizers
iv) FASTA_Nanopore files provided by EQA organizers
v) Not applicable

1.4. Which type of the test material did you use for isolate EURGen-2024-04
i) FASTQ_Illumina files provided by EQA organizers
ii) FASTQ_Nanopore files provided by EQA organizers
iii) FASTA_Illumina files provided by EQA organizers
iv) FASTA_Nanopore files provided by EQA organizers
v) Not applicable

## Questions regarding the storage and handling of live cultures (only relevant if you have used the live cultures as test material)

2.1 Please select the date when parcel with live cultures was received: [DD/MM/YYYY]

2.2 Storage conditions of the bacterial cultures (swabs) during the time between reception and processing (please select one answer):

i) -80˚C
ii) -20˚C
iii) 4˚C-8˚C
iv) Room temperature
v) No storage time
vi) Not applicable
vii) Other, please specify

2.3 Date when the processing of live bacterial cultures (BACT samples) was started

2.4 Date when the processing of live bacterial cultures (BACT samples) was finished (when cultures are ready for DNA extraction)

2.5 How were the live bacterial cultures (BACT samples) cultivated
2.5.A Type of agar media/liquid broth used:
2.5.B Incubation time (hours):
2.5.C Incubation temperature (ºC):

2.6 For EURGen-2024-01_BACT, which DNA extraction procedure was applied. Please note any deviations from the kit or cited protocol (enter 'NA' if not applicable):
2.6.A If manual extraction; kit used, full name:

2.6.B If manual extraction; catalogue number of kit:
2.6.C If manual extraction, modifications to kit protocol:
2.6.D If automatic extraction; robot used:
2.6.E If automatic extraction; specific protocol:
2.6.F If automatic extraction; modifications to protocol:
2.6.G If other, please specify:

2.7 For EURGen-2024-02_BACT, which DNA extraction procedure was applied. Please note any deviations from the kit or cited protocol (enter 'NA' if not applicable):
2.7.A If manual extraction; kit used, full name:
2.7.B If manual extraction; catalogue number of kit:
2.7.C If manual extraction, modifications to kit protocol:
2.7.D If automatic extraction; robot used:
2.7.E If automatic extraction; specific protocol:
2.7.F If automatic extraction; modifications to protocol:
2.7.G If other, please specify:

2.8 For EURGen-2024-03_BACT, which DNA extraction procedure was applied. Please note any deviations from the kit or cited protocol (enter 'NA' if not applicable):
2.8.A If manual extraction; kit used, full name:
2.8.B If manual extraction; catalogue number of kit:
2.8.C If manual extraction, modifications to kit protocol:
2.8.D If automatic extraction; robot used:
2.8.E If automatic extraction; specific protocol:
2.8.F If automatic extraction; modifications to protocol:
2.8.G If other, please specify:

2.9 For EURGen-2024-04_BACT, which DNA extraction procedure was applied. Please note any deviations from the kit or cited protocol (enter 'NA' if not applicable):
2.9.A If manual extraction; kit used, full name:
2.9.B If manual extraction; catalogue number of kit:
2.9.C If manual extraction, modifications to kit protocol:
2.9.D If automatic extraction; robot used:
2.9.E If automatic extraction; specific protocol:
2.9.F If automatic extraction; modifications to protocol:
2.9.G If other, please specify:

2.10 After DNA extraction from live cultures, how was the DNA concentration prior to library preparation measured (please select one answer)
i) Qubit® (Invitrogen™/Thermo Fisher Scientific)
ii) NanodropTM (Thermo Fisher Scientific)

iii) BioanalyzerTM (Agilent Technologies)
iv) DNA concentration not measured
v) Not applicable
vi) Other, please specify (text box)

2.11 Measurement of DNA concentration (ng/µl) for each test strain (bacterial cultures received)
2.11.A For EURGen-2024-01_BACT:
2.11.B For EURGen-2024-02_BACT:
2.11.C For EURGen-2024-03_BACT:
2.11.D For EURGen-2024-04_BACT:

2.12 Measurement of total DNA amount (ng) for each test strain (bacterial cultures received)
2.12.A For EURGen-2024-01_BACT:
2.12.B For EURGen-2024-02_BACT:
2.12.C For EURGen-2024-03_BACT:
2.12.D For EURGen-2024-04_BACT:

2.13 If relevant, measurement of DNA quality (e.g. Bioanalyser, 260/280 ratio, other) for each test strain (bacterial cultures received)
2.13.A For EURGen-2024-01_BACT:
2.13.B For EURGen-2024-02_BACT:
2.13.C For EURGen-2024-03_BACT:
2.13.D For EURGen-2024-04_BACT:

2.14 For isolate EURGen-2024-01_BACT, did you confirm the quality of the DNA through gel electrophoresis (see description in the protocol for this optional check) (not mandatory)?
i) Yes
ii) No

2.15 For isolate EURGen-2024-02_BACT, did you confirm the quality of the DNA through gel electrophoresis (see description in the protocol for this optional check) (not mandatory)?
i) Yes
ii) No

2.16 For isolate EURGen-2024-03_BACT, did you confirm the quality of the DNA through gel electrophoresis (see description in the protocol for this optional check) (not mandatory)?
i) Yes
ii) No

2.17 For isolate EURGen-2024-04_BACT, did you confirm the quality of the DNA through gel electrophoresis (see description in the protocol for this optional check) (not mandatory)?

i) Yes
ii) No

## Questions regarding the storage and handling of received DNA (only relevant if you have used DNA as test material)

3.1 Please select the date when parcel with DNA was received:

3.2 Please select the date when the processing of pre-isolated DNA was started

3.3 Please select the date when the processing of pre-isolated DNA was finished

3.4 Storage conditions of the DNA test material during the time between reception and processing (please select one answer):

i) -80˚C
ii) -20˚C
iii) 4˚C-8˚C
iv) Room Temperature
v) No storage time
vi) Not applicable
vii) Other, please specify (text box)

3.5 How was the DNA concentration measured prior to library preparation (please select one answer):

i) Qubit® (Invitrogen™/Thermo Fisher Scientific)
ii) NanodropTM (Thermo Fisher Scientific)
iii) BioanalyzerTM (Agilent Technologies)
iv) DNA concentration not measured
v) Not applicable
vi) Other, please specify (text box)

3.6 Measurement of DNA concentration (ng/µl) for each test strain (pre-isolaed DNA):

3.6.A For EURGen-2024-01_DNA:
3.6.B For EURGen-2024-02_DNA:
3.6.C For EURGen-2024-03_DNA:
3.6.D For EURGen-2024-04_DNA:

3.7 Measurement of total DNA amount (ng) for each test strain (pre-isolated DNA):

3.7.A For EURGen-2024-01_DNA:

3.7.B For EURGen-2024-02_DNA:

3.7.C For EURGen-2024-03_DNA:

3.7.D For EURGen-2024-04_DNA:

3.8 If relevant, measurement of DNA quality (e.g. Bioanalyser, 260/280 ratio, other) for each test strain (pre-isolated DNA)

3.8.A For EURGen-2024-01_DNA:

3.8.B For EURGen-2024-02_DNA:

3.8.C For EURGen-2024-03_DNA:

3.8.D For EURGen-2024-04_DNA:

3.9 For EURGen-2024-01_DNA, did you confirm the quality of the DNA through gel electrophoresis (see description in the protocol for this optional check) (not mandatory)?

i) Yes
ii) No

3.10 For EURGen-2024-02_DNA, did you confirm the quality of the DNA through gel electrophoresis (see description in the protocol for this optional check) (not mandatory)?

i) Yes
ii) No

3.11 For EURGen-2024-03_DNA, did you confirm the quality of the DNA through gel electrophoresis (see description in the protocol for this optional check) (not mandatory)?

i) Yes
ii) No

3.12 For EURGen-2024-04_DNA, did you confirm the quality of the DNA through gel electrophoresis (see description in the protocol for this optional check) (not mandatory)?

i) Yes
ii) No

# 4 Questions regarding sequencing method (only relevant if you have performed sequencing using the test material (live cultures and/or pre-isolated DNA)

4.1 Which protocol was used to prepare the libraries for sequencing? For commercial kits please provide the full kit name and catalogue number. For non-commercial kits please provide a citation for the protocol or submit a summary of the protocol. Please note any deviations from the kit or cited protocol (enter 'NA' if not applicable):

4.1.A For commercial kits; full kit name:

4.1.B For commercial kits; catalogue number:

4.1.C For noncommercial kits; citation for the protocol:

4.1.D For noncommercial kits; summary of the protocol:

4.1.E Deviations from the kit or cited protocol:

4.2 Please indicate the sequencing platform you used (please select one answer):

i) PacBio Revio® long read system (Pacific Biosciences California, USA)
ii) PacBio Sequel® long read system (Pacific Biosciences, California, USA)
iii) Nanopore MinION® (Oxford Nanopore Technologies, Oxford, United Kingdom)
iv) Nanopore GridION® (Oxford Nanopore Technologies, Oxford, United Kingdom)
v) Nanopore PromethION® (Oxford Nanopore Technologies, Oxford, United Kingdom)
vi) ABI SOLiD™ (Thermofisher Scientific, Massachusetts, USA)
vii) Genome Analyzer lix (Illumina Inc. California, USA)
viii) Genome Sequencer FLX™ System (454) (Roche Holding AG, Basel, Switzerland)
ix) Genome Sequencer FLX+™ System (454) (Roche Holding AG, Basel, Switzerland)
x) Genome Sequencer Junior™ System (454) (Roche Holding AG, Basel, Switzerland)
xi) HiScan™ SQ System (Illumina Inc. California, USA)
xii) HiSeq® 1000 (Illumina Inc. California, USA)
xiii) HISeq® 1500 (Illumina Inc. California, USA)
xiv) HiSeq® 2000 (Illumina Inc. California, USA)
xv) HiSeq® 2500 (Illumina Inc. California, USA)
xvi) HiSeq® 4000 (Illumina Inc. California, USA)
xvii) HiSeq® X (Illumina Inc. California, USA)
xviii) Ion Torrent PGM™ (Ion Torrent Systems, Inc., New Hampshire, USA)
xix) Ion Torrent Proton™ (Ion Torrent Systems, Inc., New Hampshire, USA)

xx) MGI Sequencer DNBSEQ-G400™ (MGI Tech, Shenzen, China)
xxi) MGI Sequencer DNBSEQ-G50™ (MGI Tech, Shenzen, China)
xxii) MGI Sequencer DNBSEQ-T7™ (MGI Tech, Shenzen, China)
xxiii) MiniSeq® (Illumina Inc. California, USA)
xxiv) MiSeq® (Illumina Inc. California, USA)
xxv) MiSeq® Dx (Illumina Inc. California, USA)
xxvi) MiSeq® FGx (Illumina Inc. California, USA)
xxvii) NextSeq® 500 (Illumina Inc. California, USA)
xxviii) NextSeq® 550 (Illumina Inc. California, USA)
xxix) NextSeq® 1000 (Illumina Inc. California, USA)
xxx) NextSeq® 2000 (Illumina Inc. California, USA)
xxxi) NovaSeq® 6000 (Illumina Inc. California, USA)
xxxii) Other

4.3 If the sequencing platform not listed above, please indicate the sequencing platform you used:

4.4 How much DNA per sample (ng) was used as input for library preparation?

4.5 How was the sequencing performed (please select one answer)?
i) Single-end
ii) Paired-end
iii) Not applicable

4.6 What was the expected read length (bp) before the sequencing run?

4.7 If Nanopore sequencing was performed, select the flowcell that was used.
i) MinION/GridION flowcell
ii) PromethION flowcell
iii) Flongle flowcell

4.8 If Nanopore sequencing was performed, select the flowcell chemistry that was used.
i) R9.4.1
ii) R10.4
iii) R10.4.1

4.9 For base calling, which tool, version, and method was used (e.g. guppy version 4.4.0 high accuracy)?

## Questions regarding quality control of sequence data (relevant if you used live cultures and/or pre-isolated DNA, or FASTQ/FASTQ as test material)

5.1 How was the quality control (QC) of raw data performed (e.g., FASTQC analyses)?

5.2 Were the reads trimmed before bioinformatics analysis ? (Note; this question refers to trimming performed actively by the participant (i.e. if trimming is performed automatically by your sequencing machine, this does not apply to this question)

i) Yes
ii) No

5.3 If reads were trimmed, which software was used (please insert name, version and URL)?

5.4 Were the reads filtered before bioinformatics analysis ? (This question refers to filtering performed actively by the participant. If filtering is performed automatically by your sequencing machine, the question does not apply)

i) Yes
ii) No

5.5 If reads were filtered, which software was used (please insert name, version and URL)?

5.6 If applicable, which assembly tool did you use to assemble the reads (please insert the name, version number and URL (e.g., SPAdes, version 3.15.4, https://cab.spbu.ru/software/spades/)?

5.7 How was the QC of assembly performed (please mention the program used (e.g., QUAST) and include the results such as N50 and L50 values)?

## Questions regarding species identification (relevant if you used live cultures and/or pre-isolated DNA, or FASTQ/FASTQ as test material)

6.1 Which species did you detect for EURGen-2024-01?

i) E. coli
ii) Klebsiella pneumoniae
iii) Acinetobacter baumannii
iv) Pseudomonas aeruginosa
v) No results submitted

6.2 Which species did you detect for EURGen-2024-02?

i) E. coli
ii) Klebsiella pneumoniae
iii) Acinetobacter baumannii
iv) Pseudomonas aeruginosa
v) No results submitted

6.3 Which species did you detect for EURGen-2024-03?

i) E. coli
ii) Klebsiella pneumoniae
iii) Acinetobacter baumannii
iv) Pseudomonas aeruginosa
v) No results submitted

6.4 Which species did you detect for EURGen-2024-04?

i) E. coli
ii) Klebsiella pneumoniae
iii) Acinetobacter baumannii
iv) Pseudomonas aeruginosa
v) No results submitted

## Questions regarding prediction of Multi Locus Sequence Types (relevant if you used live cultures and/or pre-isolated DNA, or FASTQ/FASTQ as test material)

7.1 Pipeline type: local or web-based pipeline:

7.2 Software: publicly available software, commercial software, or in-house scripts. If you used a software, please report the name and the version number. If you used an in-house script, please specify the program

7.3 Database: publicly available database, commercial database, or an in-house database. If you used an available database, please report database name and version number. If you used an in-house database, please specify the loci included in the scheme

7.4 Parameters of the software: default parameters or defined by you. If you used parameters defined by you, please specify them

7.5 If applicable, please specify the URL of the software and/or database used

## Questions regarding detection of plasmid replicons (relevant if you used live cultures and/or pre-isolated DNA, or FASTQ/FASTQ as test material)

8.1 Pipeline type: local or web-based pipeline

8.2 Software: publicly available software, commercial software, or in-house scripts. If you used a software, please report the name and the version number. If you used an in-house script, please specify the program.

8.3 Database: publicly available database, commercial database, or an in-house database. If you used an available database, please report database name and version number. If you used an in-house database, please briefly describe the sequences included in the database

8.4 Parameters of the software: default parameters or defined by you. If you used parameters defined by you, please specify them (e.g. minimum length 80% and minimum identity 95%, etc.)

8.5 If applicable, please specify the URL of the software and/or database used

## Questions regarding prediction of AMR genes (relevant if you used live cultures and/or pre-isolated DNA, or FASTQ/FASTQ as test material)

9.1 Pipeline type: local or web-based pipeline

9.2 Software: publicly available software, commercial software, or in-house scripts. If you used a software, please report the name and the version number. If you used an in-house script, please specify the program.

9.3 Database: publicly available database, commercial database, or an in-house database. If you used an available database, please report database name and version number. If you used an in-house database, please briefly describe the genes included in the database

9.4 Parameters of the software: default parameters or defined by you. If you used parameters defined by you, please specify them (e.g. minimum length 80% and minimum identity 95%, etc.)

9.5 If applicable, please specify the URL of the software and/or database used

## Questions regarding detection of chromosomal mutations mediating AMR (relevant if you used live cultures and/or pre-isolated DNA, or FASTQ/FASTQ as test material)

10.1 Pipeline type: local or web-based pipeline

10.2 Software: publicly available software, commercial software, or in-house scripts. If you used a software, please report the name and the version number. If you used an in-house script, please specify the program

10.3 Database: publicly available database, commercial database, or an in-house database. If you used an available database, please report database name and version number. If you used an in-house database, please briefly describe the point mutations included in the database

10.4 Parameters of the software: default parameters or defined by you. If you used parameters defined by you, please specify them (e.g. minimum length 80% and minimum identity 95%, etc.)

10.5 If applicable, please specify the URL of the software and/or database used

## Questions regarding WGS-based prediction of AMR profiles (relevant if you used live cultures and/or pre-isolated DNA, or FASTQ/FASTQ as test material)

11.1 Pipeline type: local or web-based pipeline

11.2 Software: publicly available software, commercial software, or in-house scripts. If you used a software, please report the name and the version number. If you used an in-house script, please specify the program

11.3 Database: publicly available database, commercial database, or an in-house database. If you used an available database, please report database name and version number. If you used an in-house database, please briefly describe the sequences included in the database

11.4 Parameters of the software: default parameters or defined by you. If you used parameters defined by you, please specify them (e.g. resistance is called if gene is present with minimum length 100% and minimum identity 98%, etc.)

11.5 If applicable, please specify the URL of the software and/or database used

## Questions regarding sequencing data (FASTQ files) submitted by participants for quality control (relevant if you used live cultures and/or pre-isolated DNA as test material)

12.1 Have the FASTQ files (raw, non-assembled sequence data, file names as indicated in the protocol) been uploaded to the ScienceData-folder for EURGen-2024-01_BACT?

i) Yes
ii) No

12.2 Have the FASTQ files (raw, non-assembled sequence data, file names as indicated in the protocol) been uploaded to the ScienceData-folder for EURGen-2024-02_BACT?

i) Yes
ii) No

12.3 Have the FASTQ files (raw, non-assembled sequence data, file names as indicated in the protocol) been uploaded to the ScienceData-folder for EURGen-2024-03_BACT?

i) Yes
ii) No

12.4 Have the FASTQ files (raw, non-assembled sequence data, file names as indicated in the protocol) been uploaded to the ScienceData-folder for EURGen-2024-04_BACT?

i) Yes
ii) No

12.5 Have the FASTQ files (raw, non-assembled sequence data, file names as indicated in the protocol) been uploaded to the ScienceData-folder for EURGen-2024-01_DNA?

i) Yes

ii) No

12.6 Have the FASTQ files (raw, non-assembled sequence data, file names as indicated in the protocol) been uploaded to the ScienceData-folder for EURGen-2024-02_DNA?

i) Yes
ii) No

12.7 Have the FASTQ files (raw, non-assembled sequence data, file names as indicated in the protocol) been uploaded to the ScienceData-folder for EURGen-2024-03_DNA?

i) Yes
ii) No

12.8 Have the FASTQ files (raw, non-assembled sequence data, file names as indicated in the protocol) been uploaded to the ScienceData-folder for EURGen-2024-04_DNA?

i) Yes
ii) No

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