Protocol for the EURGen-RefLabCap External Quality Assessment exercise 2023

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1. Overview and objectives

External Quality Assessment (EQA) exercise is an important tool 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 2023 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 2023 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 WS1 and WS2 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 current EQA exercise focuses on *Escherichia coli*, *Klebsiella pneumoniae, Acinetobacter baumannii* and *Pseudomonas aeruginosa* and for each test isolate, the provided test material is pre-isolated DNA (Table 1). Participants are invited to proceed with sequencing the pre-isolated DNA by either short-read or long-read sequencing technology, using the routine methods and protocols currently implemented in their laboratories. If, for some reason, NRLs cannot sequence the DNA at their facility, they can request the raw sequencing data corresponding to the test material (FASTQ or FASTA files, either from short-read or long-read sequencing), via the registration form.

Participating laboratories nominated for WS1 are provided with DNA from two isolates for testing, i.e., the two test codes belonging to the species included in WS1. Participating laboratories nominated for both WS1 and WS2 are provided with DNA from all four isolates for testing. Information on the methods used to extract the DNA and to generate the EURGen-RefLabCap EQA 2023 test sequence data is available in Appendix 1.

Table 1: Codes for test material included in the EURGen-RefLabCap EQA 2023.

Sample no.	Material test code	Material type
1	EURGen-2023-01	DNA
2	EURGen-2023-02	DNA
3	EURGen-2023-03	DNA
4	EURGen-2023-04	DNA

3. Outline of the EURGen-RefLabCap EQA 2023

3.1. Comparison with the previous EQA

The 2023 EQA is conducted similarly to the EURGen-RefLabCap 2022 EQA. The main differences are the receipt of DNA for sequencing (instead of receiving sequence data) and the inclusion of pathogens belonging to WS1 and WS2 (instead of only those belonging to WS1). In EQA 2023, we will also ask the participants to share the raw sequencing data that they have generated with the EQA 2023 organizers. 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 March 2023, contact persons from the EURGen-RefLabCap project received the pre-notification and instructions for registration of the EURGen-RefLabCap EQA 2023. Those who registered their laboratory to participate in the EURGen-RefLabCap EQA 2023 will receive the test material (dehydrated DNA) in June 2023. 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 DNA in their settings 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. Rehydration and storage of DNA

Each Eppendorf tube of the pre-isolated DNA contains a minimum of 125 ng of dehydrated genomic DNA. Before use, the supplied DNA should be rehydrated:

- Add 50 µL (microliters) nuclease free water or aqueous buffer to the dried 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);
- Incubate the 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 checked and visualized by agarose gel electrophoresis. The amount of DNA supplied in each tube is sufficient to run a small fraction on a 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.

Table 2: Bioinformatics results requested to be submitted for EURGen-RefLabCap EQA 2023.

	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 2023 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. Intrinsic genetic determinants and intrinsic AMR profiles are not part of expected results and will be scored as incorrect 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*' (see https://www.eurgen-reflabcap.eu/resources/wgs-tools) is suggested, though, the participants may choose to use their own WGS analysis set-up currently implemented at their laboratory. For the analyses of C/CRAb and C/CRPa, guidance on recommended protocols and bioinformatics approaches is also provided in the document '*Proposed common WGS-based genome analysis methods and standard protocols for national surveillance and integrated outbreak investigations of Pseudomonas aeruginosa and Acinetobacter baumannii*' (https://www.eurgen-reflabcap.eu/resources/protocols-and-guidelines**)**, though, participating laboratories can choose to apply any tools they find suitable for the analyses. 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 EQA 2023, 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;

i) raw sequencing data (FASTQ files) generated by participating laboratories using short read and/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) bioinformatics results via the EURGen-RefLabCap EQA 2023 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 template provided 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 must indicate if results were based on pre-isolated DNA (including how the DNA was sequenced), or if they used sequence data received from the EQA providers (i.e., 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.

Results related to antimicrobial resistance (AMR)

All detected genes 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 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).

For results related to AMR (detection of genetic determinants mediating AMR and *in silico* prediction of AMR profiles), please note that the analysis might require collaboration between a bioinformatician and a microbiologist with knowledge within the field of AMR.

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 ResFinder) and Achtman scheme for *E. coli* (*E. coli*#1, if using ResFinder). Table 5 shows the seven alleles of the four bacterial species covered in the EQA 2023. 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 STs.

Table 5. Alleles included in the multi-locus sequence typing (MLST) schemes relevant for EQA 2023

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 baumanii (Oxford scheme)	cpn60	gdhB	gltA	gpi	gyrB	recA	rpoD
Pseudomonas aeruginosa	acsA	aroE	guaA	mutL	nuoD	ppsA	trpE

4.2. 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 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 related to EURGen-2023-01 should be named as EURGen-RLC-0XX_EURGen-2023-01_R1.fastq.gz and EURGen-RLC-0XX_EURGen-2023-01_R2.fastq.gz, while single-end FASTQ files should be named as EURGen-RLC-0XX_EURGen-2023-01.fastq.gz. 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).

Upon uploading the files on ScienceData, please make sure that the size of the files corresponds to the expected file size. For detailed information on how to upload files 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 check 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.

4.3. Deadline for submission of results

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 15 August 2023 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 (use 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 2023, participants are kindly 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 report presenting their evaluated 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 assembled and run through a QC pipeline by the EQA 2023 organizers. The pipeline analyses the quality of sequences generated by Illumina as well as Oxford Nanopore technology to the extent possible. The QC analysis will evaluate several parameters, including (but not limited to) number of reads, number of contigs, coverage, N50, MLST type and Q-score.

When receiving the results evaluation, each participant is asked to assess their own performance and consider whether the obtained results should lead to adjustments internally, considering their handling of bacterial strains and/or DNA sequencing.

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 acknowledged in the publications. 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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PROTOCOL for EURGen-RefLabCap EQA, 2023 – APPENDICES

Appendix 1 Methods used to generate the EURGen-RefLabCap EQA 2023 test material (DNA, FASTA and FASTQ sequences)

Appendix 2 Using ScienceData platform to transfer sequencing files

Appendix 3 EURGen-RefLabCap EQA 2023 webtool manual

Appendix 4 Testforms – overview of method information to be submitted via the webtool

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Appendix 1

Methods used to generate the EURGen-RefLabCap EQA 2023 test material (DNA, FASTA and FASTQ sequences)

	Dehydrated DNA
DNA extraction kit	Invitrogen™ Easy-DNA™ gDNA Purification Kit
DNA extraction protocol	In house modified protocol based on Invitrogen™ Easy-DNA™ Kit user guide

	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	In house modified protocol based on Illumina® Nextera XR DNA Library Prep Reference Guide	Rapid sequencing DNA V14 - barcoding protocol
How QC of sequence data was performed	FastQC v0.11.5	NanoStat v1.4.0
How assembly of sequence data was performed	Spades v3.11.0	Flye v2.9.2

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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 evaluation
- Downloading of sequencing data (FASA 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 EQA2023 protocl for naming sequencing data files.

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

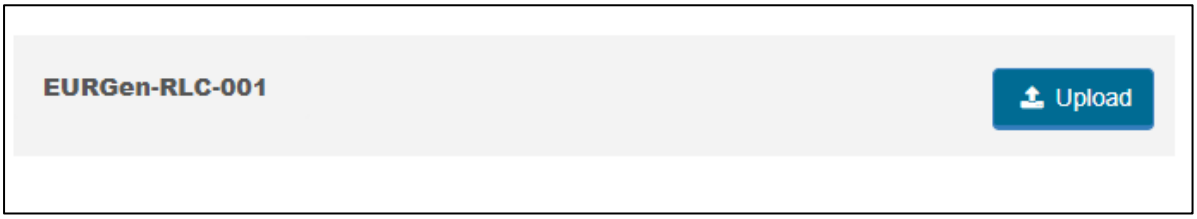

Figure 1. Upload page on ScienceData platform

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

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

Downloading files to 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 2.

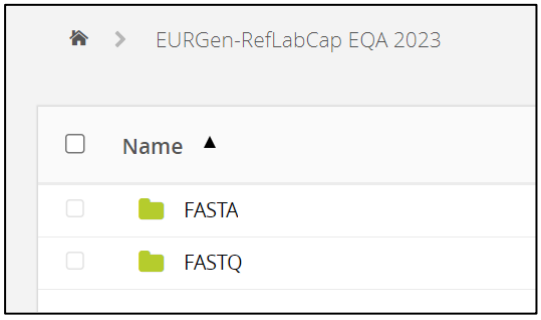

Image 2. FASTA and FASTQ folders in ScienceData platform

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 sequences)

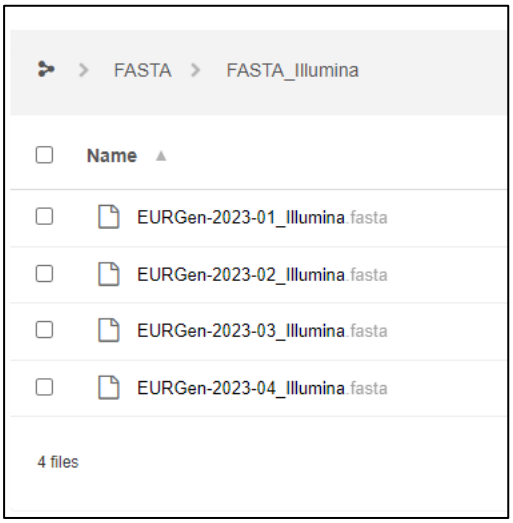

Image 1. FASTA files located in FASTA_Illumina subfolder

b) Subfolder FASTA_Nanopore (4 sequences)

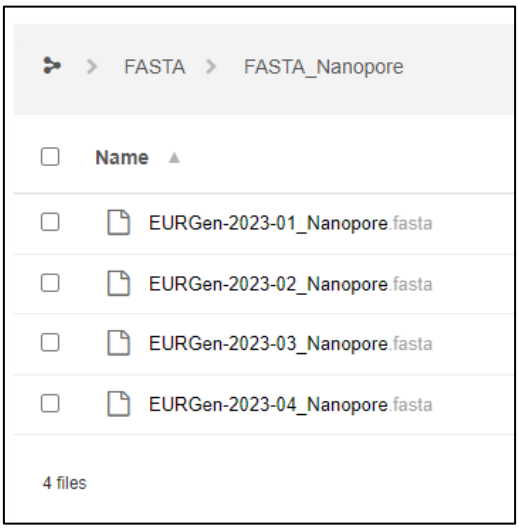

Image 4. FASTA files located in FASTA_Nanopore subfolder

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 sequences)

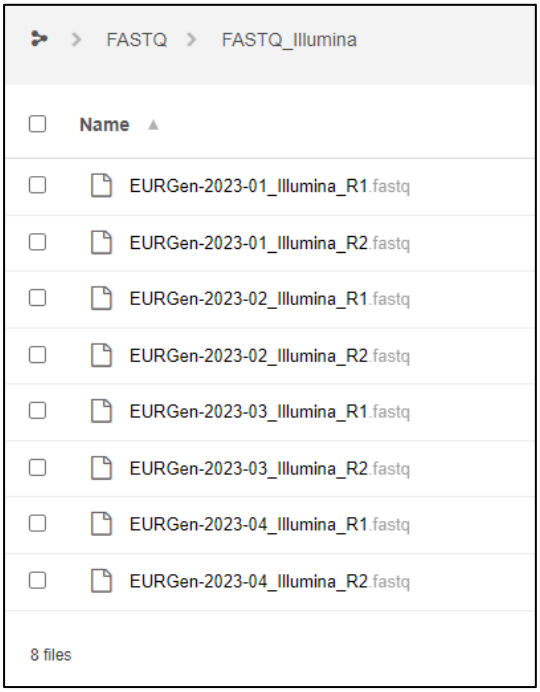

Image 5. FASTQ files located in FASTQ_Illumina subfolder

b) Subfolder FASTQ_Nanopore (4 sequences)

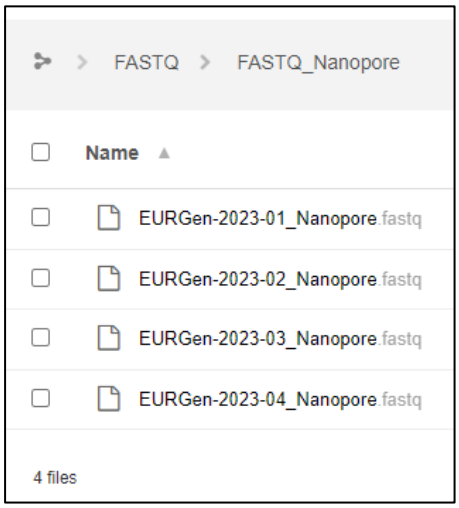

Image 6. FASTQ files located in FASTQ_Nanopore subfolder

4. To download, select the files you want to download and click the "download" button as shown in Image 7.

Figure 7. Downloading files from ScienceData

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Appendix 3

EURGen-RefLabCap EQA 2023 webtool manual

Browser requirements

IMPORTANT: The system works with the following browsers

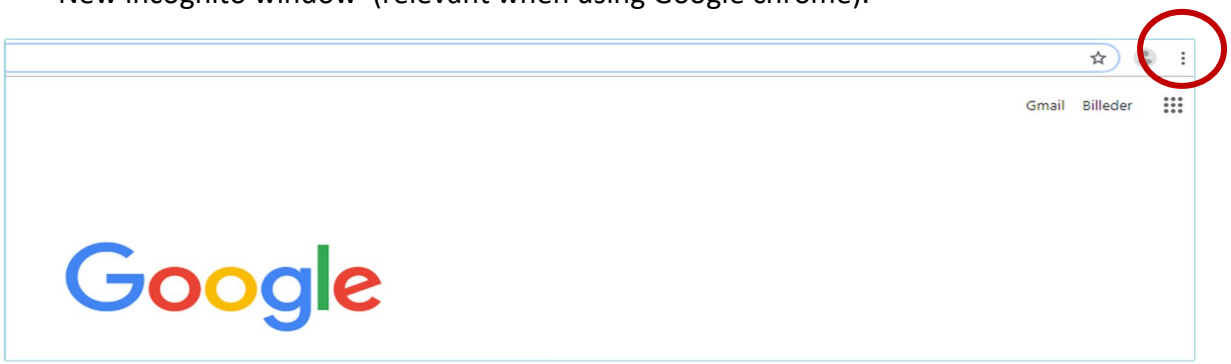
Browser	Oldest supported version*
Google Chrome	44.0
Firefox	39.0

* latest version is recommended

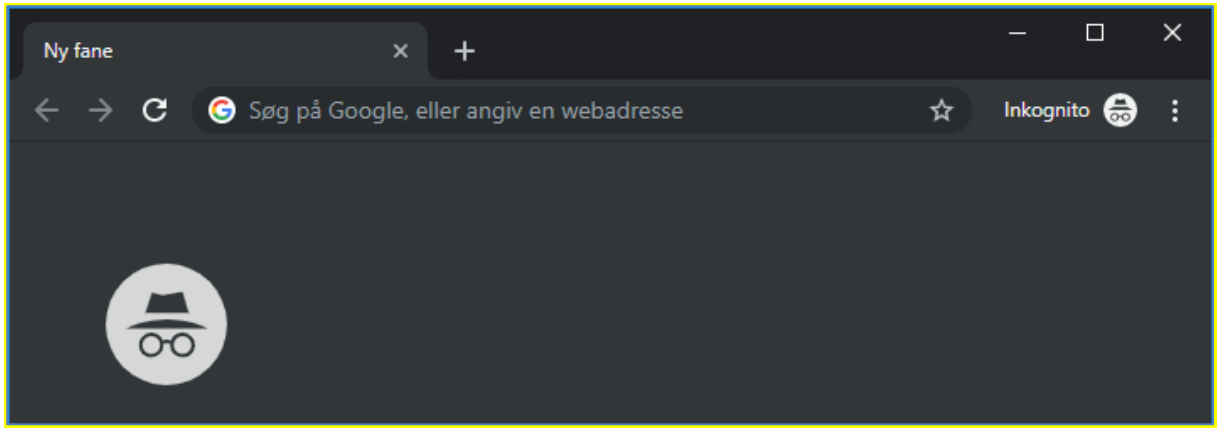
Accessing the webtool

IMPORTANT: To access the webtool, you must use an incognito window.
If you have issues with opening an incognito window, please contact the EQA Coordinator (suska@food.dtu.dk) directly.

- Open 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 (relevant when using Google chrome):

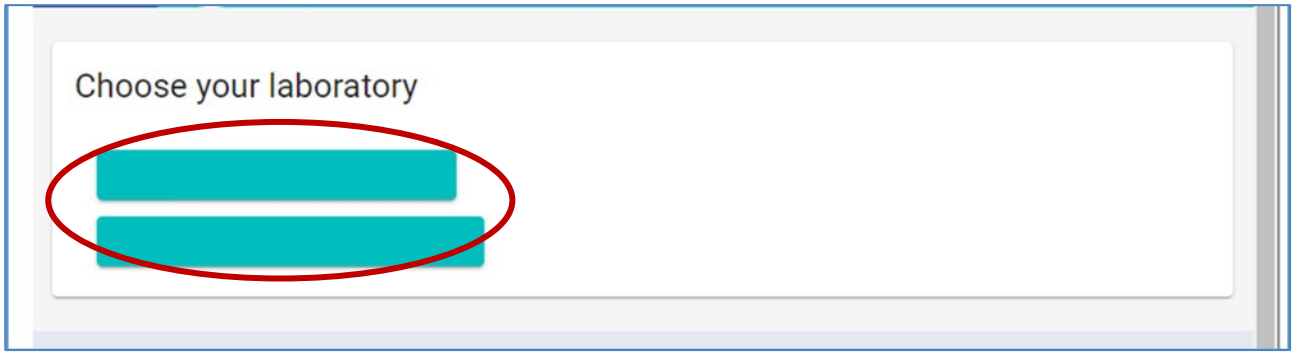


- To access the webtool, copy and paste the following link to the address bar in the incognito window and press **Enter** on the keyboard. You will reach the webtool *Sign-in* page as shown in the image below.

  Webtool link: https://eurgen-reflabcap-pt.dtu.dk

- 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.

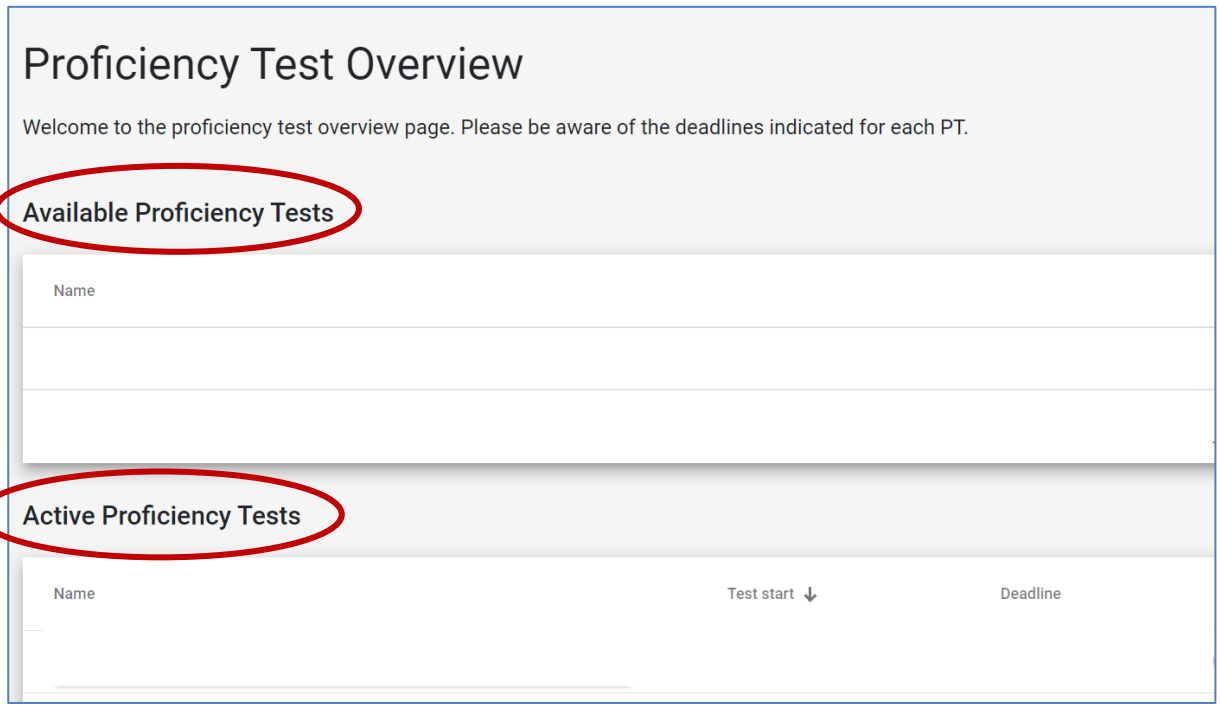


If you are connected to more than one specific laboratory, you will need to select the specific laboratory that you intend to submit results for.

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

Under "Available Proficiency Tests", sign up to the "EURGen-RefLabCap EQA 2023". The EQAs 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

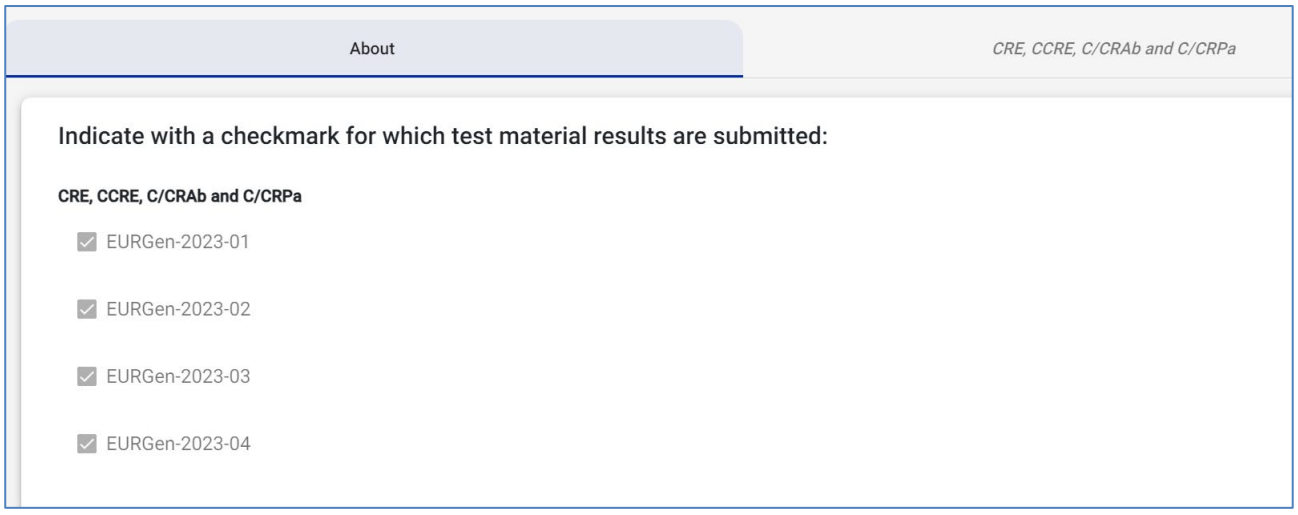
When reporting results/data in the webtool, various tabs are available:

1. **'About' tab**. With a checkmark, select the test material for which you wish to submit results, i.e., select each relevant codes
2. **'Method' tab**. Enter information regarding the sequencing and bioinformatics analyses performed
3. **'AMR' tab**. Enter results regarding antimicrobial resistance genes, chromosomal mutations mediating antimicrobial resistance and predicted antimicrobial resistance profile
4. **'MLST' tab**. Enter data regarding sequence types and alleles
5. **'Replicon' tab**. Enter data regarding plasmid replicon type

Enter data

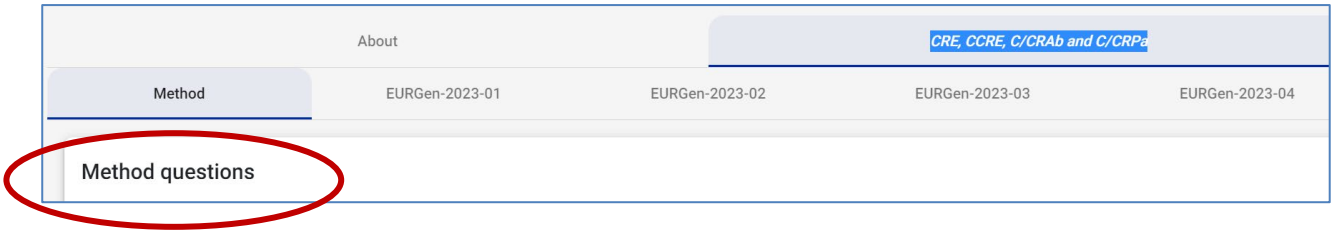
Ad 1. 'About' tab

Indicate for which test material results will be submitted for, regardless of whether DNA or raw sequencing files were used to generate the results. 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.

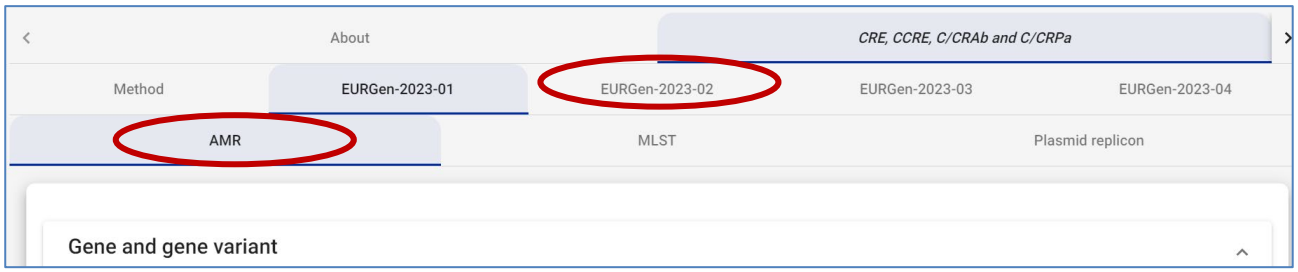


Ad 2. 'Method' tab

Respond to the questions in the 'Method' tabs.



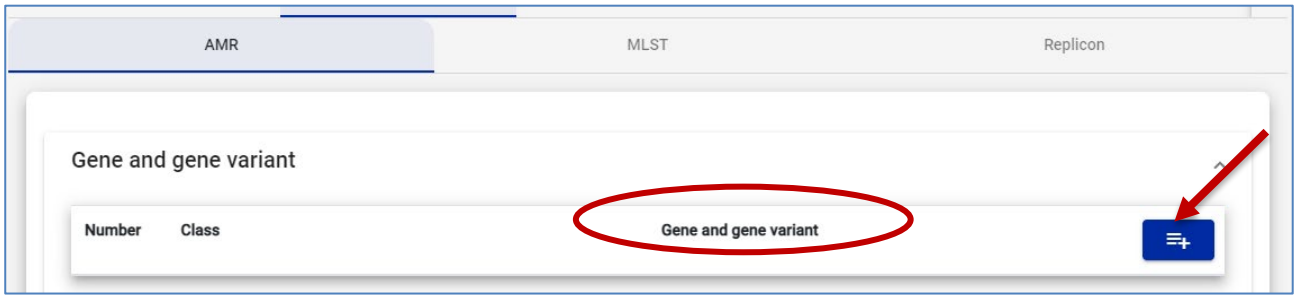
Select the tab related to one of the test materials (e.g., EURGen-2023-01). This opens access to additional tabs i.e., "AMR", "MLST", and "Plasmid replicon".



Ad 3. 'AMR' tab:

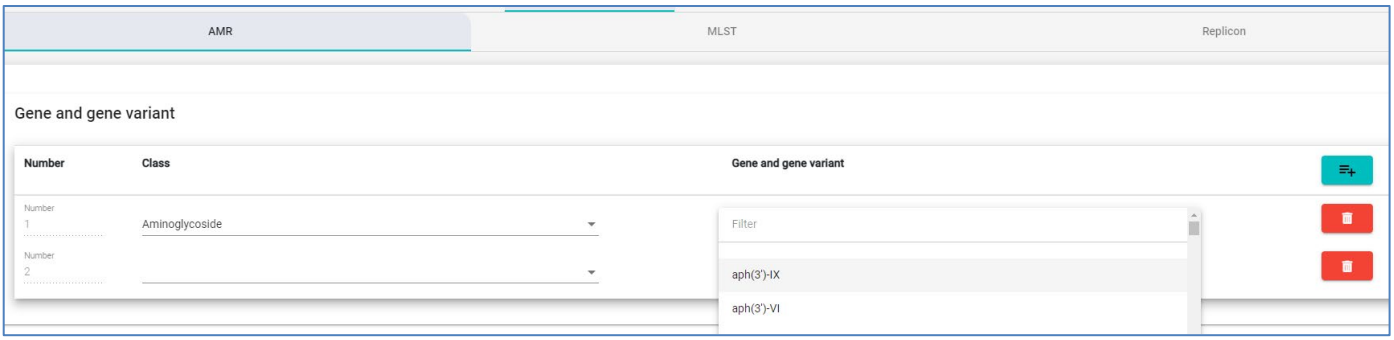
Under the AMR-tab, results related to 1) identified antimicrobial resistance genes, 2) identified chromosomal mutations mediating antimicrobial resistance, 3) and identified prediction of the antimicrobial resistance profile are uploaded.

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 antimicrobial resistance 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 #1: Antimicrobial resistance towards a limited number of antimicrobial classes is considered in this EQA. Antimicrobial classes represented in the EURGen-RefLabCap EQA 2023, and consequently in the webtool drop down list are:

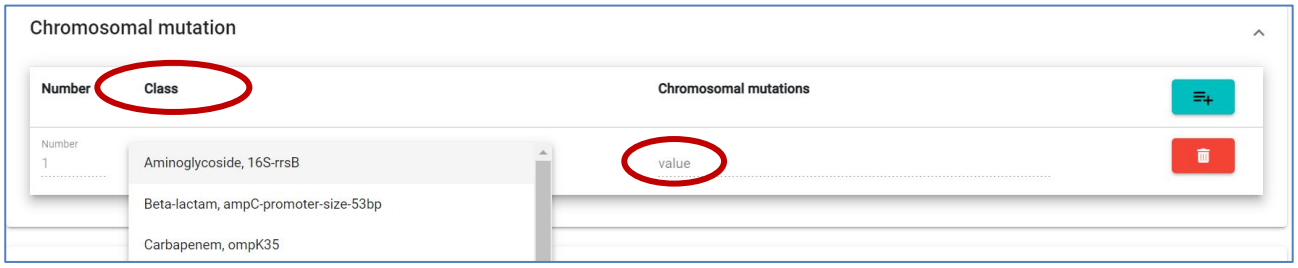
Included classes and genes for the AMR component are as presented in Table 3 and Table 4 in the EQA 2023 protocol. All genes conferring resistance to the antimicrobials in this list should be reported, *i.e.*, including genes associated with acquired resistance.

Some antimicrobial resistance 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.

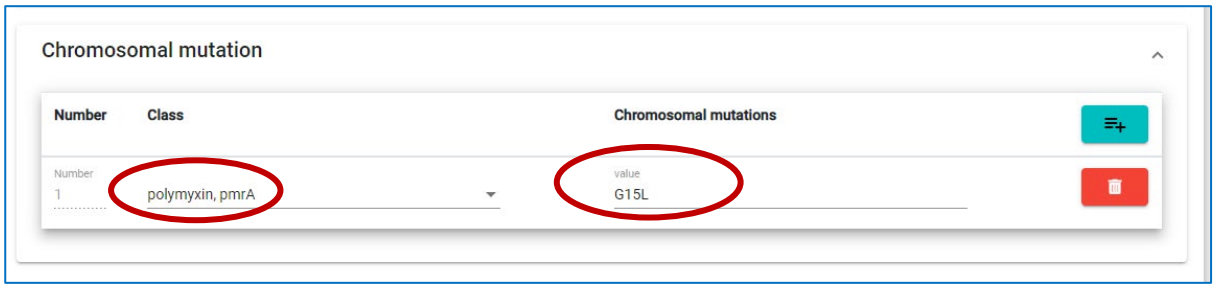
Ensure that no empty lines are saved for evaluation by clicking on the bin if you by mistake added one too many.

'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:

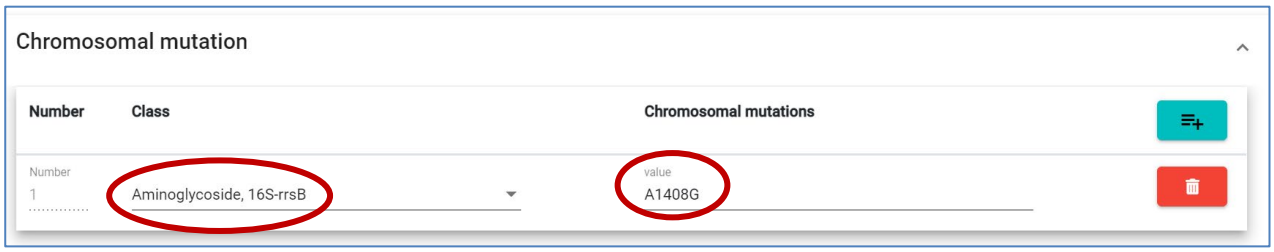
1) Indicate the reference codon (an amino acid letter, or a nucleotide letter for 16S or 23S sequences)
2) Indicate the position of the codon (a numeric value)
3) Indicate the resistance codon (amino acid letter(s), or nucleotide(s) for 16S or 23S sequences)



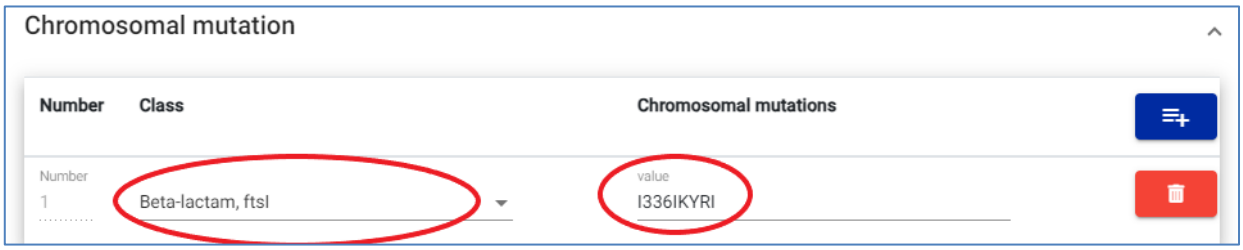
Example 1: Reporting a mutation in the *pmr*A gene which has changed the *amino acid* glycine (G) to Leucine (L) at position 15. This results in resistance to colistin that belongs to the polymyxin class of antimicrobials. Therefore, from the dropdown list under 'Class', select the 'polymyxin, *pmr*A'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



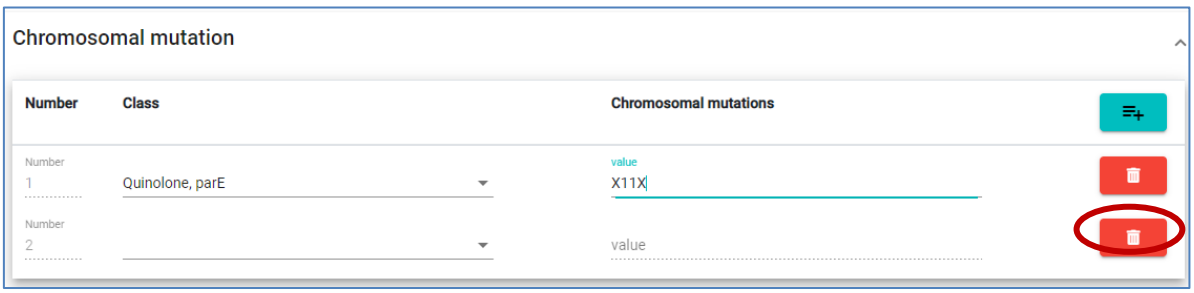
Example 2: If the mutation is in a 16S rRNA gene please select the class of antimicrobial and associate gene (e.g., Aminoglycoside, 16S-rrsB) from the dropdown menu. Hereafter, in the 'value', write the letter of the original reference *nucleotide* (A, T, C or G) and its position, followed by the new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



Example 3: If you want to report a mutation where multiple amino acids have been inserted into the 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".

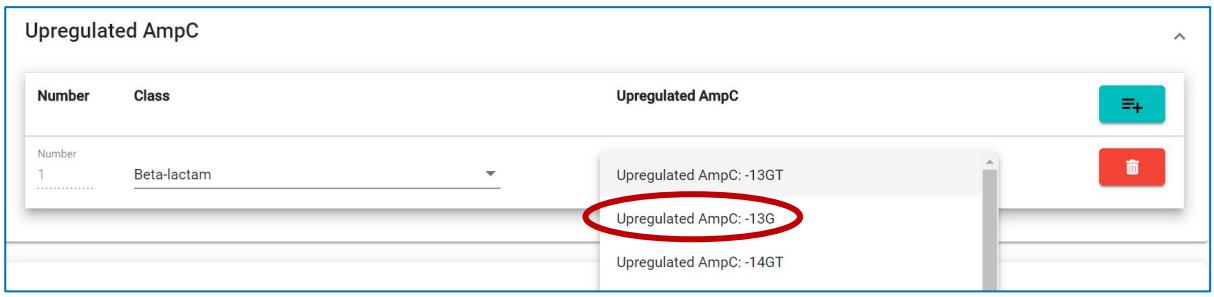


Important! Please make sure that no empty lines are saved for evaluation. If you have an empty entry, delet the entry by clicking on the "bin" button as shown in the image below.

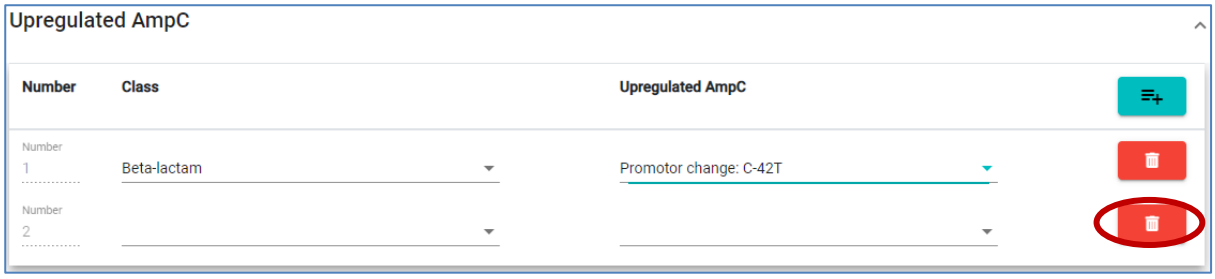


'Upregulated ampC': Upregulated ampC resistance can be reported by selecting the 'Upregulated ampC' option under the 'AMR' tab. For the 'Upregulated ampC' option, select 'Beta-lactam' under 'Class' and hereafter, from the dropdown menu under 'Upregulated ampC', select the specific mutations in the promoter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

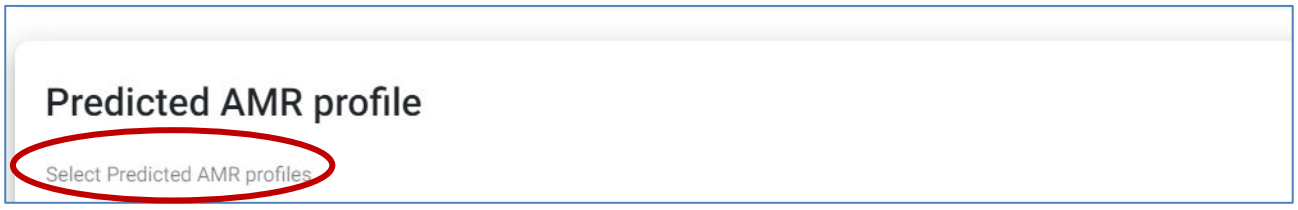
The mutations are shown in the same way as previously described for 16S and 23S sequence mutations, i.e., the reference codon is followed by a numeric value, and then followed by the resistance codon (unless the mutation is an insertion). Since the promoter is located upstream to the open reading frame (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.

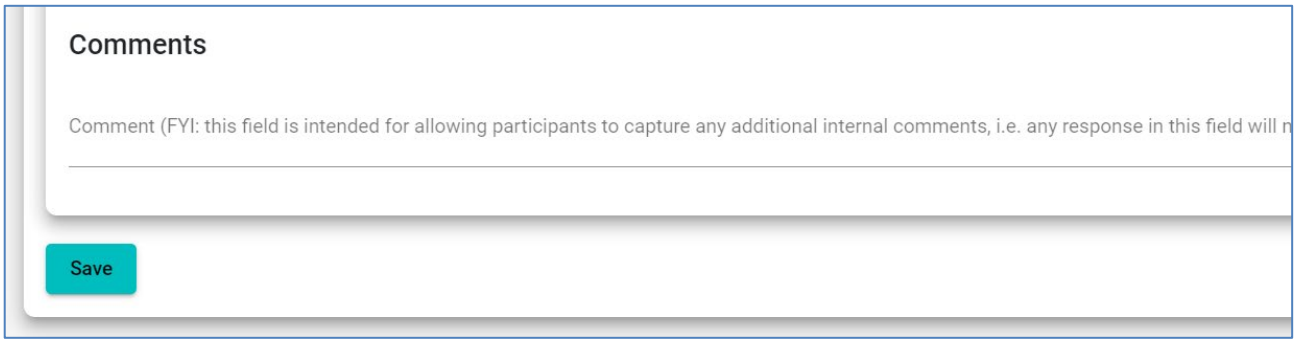


'Predicted AMR profile": Click on 'Predicted AMR profile" to select the antimicrobial agents that test strain is resistant to, based on the *in silico* prediction using sequencing data. Note that in relation to predicted AMR profile, antimicrobial resistance towards a limited number of antimicrobials is considered in this EQA. Antimicrobials represented in the EURGen-RefLabCap EQA 2023 relevant for workstream 1 and workstream 2 pathogens are listed in Table 3 and Table 4 of the EQA 2023 protocol, respectively.



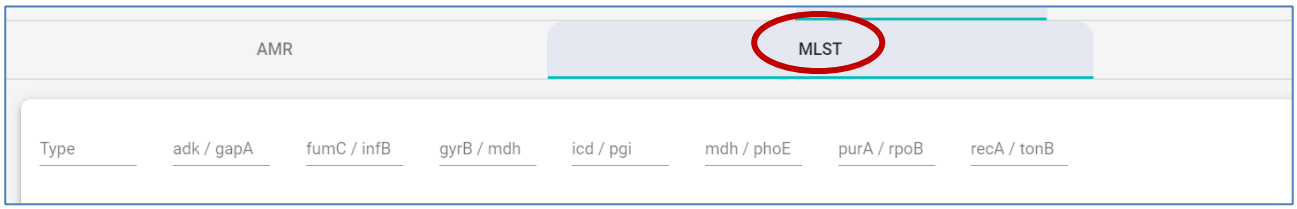
'Comments': Any comments related to the submission of the results are welcome. You may for example indicate mutations that have unknown effect on antimicrobial resistance. You may also report genes and/or mutations that were not listed in the drop-down menu of the tabs described above. Note, however,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

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



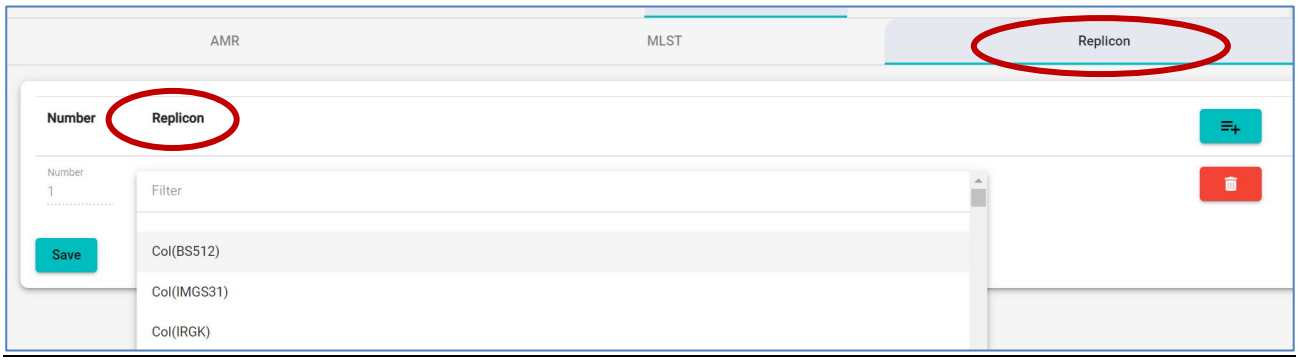
Ad 4. 'MLST' tab

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 2023 protocol (section 4.1) for information regarding MLST schemes and alleles relevant for current EURGen-RefLabCap EQA 2023.



Ad 5. 'Replicon' tab

Enter data regarding plasmid replicon type by selecting from the dropdown list.

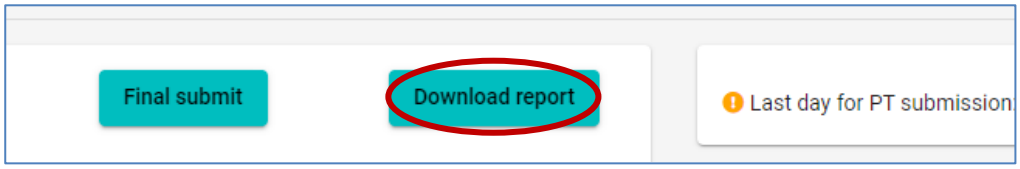


Save data

Data are saved when you click the *save* button on each page. Moreover, data are 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.

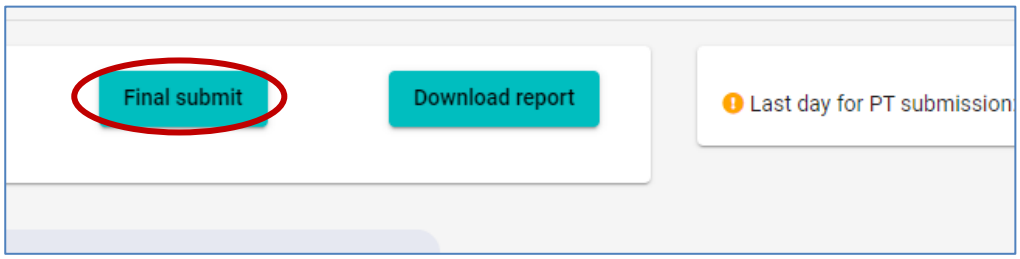


Before you have finally submitted your results (and before deadline), the database allows you to return to any test form where you can make changes to the reported data.

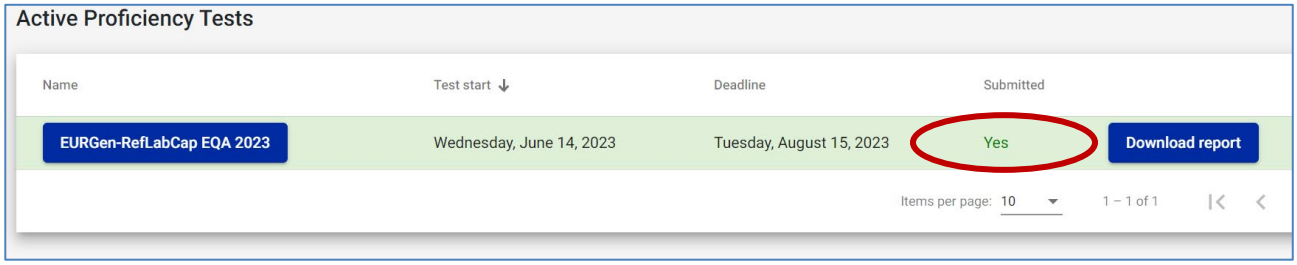
Submit data

For all test materials of the EURGen-RefLabCap EQA 2023, all uploaded data are submitted in one go.

When all information and data have been entered and revised for, 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:

Reported antimicrobial resistance genes, for a given test material, will be compared to the expected results. Each reported gene will be scored individually. When a submitted antimicrobial resistance gene is on the list of expected antimicrobial resistance genes (i.e., if the obtained and expected antimicrobial resistance genes match), a score of '1' will be achieved, whereas a mismatch (obtained result is not expected) is scored with '0'. Expected antimicrobial genes 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 antimicrobial resistance for a given test strain genome, will be compared to the expected results. Each reported mutation will be scored individually. When a submitted chromosomal mutation mediating antimicrobial resistance is on the list of expected chromosomal mutations mediating antimicrobial resistance (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'. Expected chromosomal mutations mediating antimicrobial resistance 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 antimicrobial resistance profile for a given test strain genome, will be compared to the expected results. In the EQA 2023, 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'.

The reported Multi Locus Sequence Type (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'. 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.

When the score is released and the evaluation reports are accessible, all participating laboratories will receive an email message 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).

See also the top right corner of all pages in the webtool to find the name and email address for the EQA organizer. Find also link to the EURGen-RefLabCap website (to access the relevant EQA protocol) as well as access to the current webtool manual.

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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 EQA2023
- Storage and handling of test material (relevant if you received DNA samples for sequencing)
- DNA pre-processing (relevant if you have chosen to sequence DNA samples)
- Sequencing method (relevant if you have chosen to sequence DNA samples)
- Analyses of sequencing data: multi-locus sequence types, plasmid replicons, antimicrobial resistance (AMR) genes and chromosomal point mutations mediating AMR, *in silico* AMR profiles
- Analyses of sequencing data uploaded by EQA participants

In the EURGen-RefLabCap EQA 2023, laboratories are provided with the pre-isolated DNA as test material. If, for some reason, your laboratory cannot sequence the DNA, FASTA/FASTQ files corresponding to the test isolates can be provided as test material. In the webtool, the laboratories must indicate the type of test material (DNA or sequence data, specifically FASTQ or FASTA files produced through short-read or long-read sequencing) they have used for the analyses, or if results for the specific test material code is not submitted.

1) Questions regarding test material used for EURGen-RefLabCap EQA2023

a) Which type of test material was used for EURGen-2023-01?
   i) DNA
   ii) FASTQ files
   iii) FASTA files

b) Which type of test material was used for EURGen-2023-02?
   i) DNA
   ii) FASTQ files

iii) FASTA files

c) Which type of test material was used for EURGen-2023-03?
   i) DNA
   ii) FASTQ files
   iii) FASTA files

d) Which type of test material was used for EURGen-2023-04?
   i) DNA
   ii) FASTQ files
   iii) FASTA files

2) Questions regarding DNA sample storage and handling (only relevant if you received DNA samples for sequencing)

a) Please enter the date parcel with DNA was received: [DD/MM/YYYY]

b) Storage conditions of the DNA samples in 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) Not applicable
   vi) No storage time (sequenced immediately on arrival)

3) Questions regarding DNA pre-processing (only relevant if you have chosen to sequence DNA samples)

a) How was the DNA concentration measured prior to library preparation (please select one answer)?
   i) Qubit® (Invitrogen™/Thermo Fisher Scientific)
   ii) Nanodrop™ (Thermo Fisher Scientific)
   iii) Bioanalyzer™ (Agilent Technologies)
   iv) DNA concentration not measured
   v) Not applicable
   vi) If other, please define:

b) For EURGen-2023-01, did you confirm the quality of the DNA through gel electrophoresis?
   i) Yes
   ii) No

c) For EURGen-2023-02, did you confirm the quality of the DNA through gel electrophoresis?
   i) Yes
   ii) No

d) For EURGen-2023-03, did you confirm the quality of the DNA through gel electrophoresis?
   i) Yes
   ii) No

e) For EURGen-2023-04, did you confirm the quality of the DNA through gel electrophoresis?
   i) Yes
   ii) No

4) Results of DNA concentration (ng/µl) for each test sample:
   a) For EURGen-2023-01:
   b) For EURGen-2023-02:
   c) For EURGen-2023-03:
   d) For EURGen-2023-04:

5) Results of the total DNA amount (nanograms (ng)) for each test sample (for the DNA received):
   a) For EURGen-2023-01:
   b) For EURGen-2023-02:
   c) For EURGen-2023-03:
   d) For EURGen-2023-04:

6) How was the DNA quality assessed (e.g. Nanodrop, Bioanalyzer, other) for each test sample?
   a) For EURGen-2023-01:
   b) For EURGen-2023-02:
   c) For EURGen-2023-03:
   d) For EURGen-2023-04:

7) Results of DNA quality assessment (260/280 ratio) for each test sample (for DNA received):

a) For EURGen-2023-01:
b) For EURGen-2023-02:
c) For EURGen-2023-03:
d) For EURGen-2023-04:

8) Questions regarding sequencing (relevant if you received DNA samples for sequencing)

a) Which protocol was used to prepare the sample library 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):

   i) For commercial kits; full kit name:
   ii) For commercial kits; catalogue number:
   iii) For noncommercial kits; citation for the protocol:
   iv) For noncommercial kits; summary of the protocol:
   v) Deviations from the kit or cited protocol:

b) 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) ABI SOLiD™ (Thermofisher Scientific, Massachusetts, USA)
   v) Genome Analyzer Iix (Illumina Inc. California, USA)
   vi) Genome Sequencer FLX™ System (454) (Roche Holding AG, Basel, Switzerland)
   vii) Genome Sequencer FLX+™ System (454) (Roche Holding AG, Basel, Switzerland)
   viii) Genome Sequencer Junior™ System (454) (Roche Holding AG, Basel, Switzerland)
   ix) HiScan™ SQ System (Illumina Inc. California, USA)
   x) HiSeq® 1000 (Illumina Inc. California, USA)
   xi) HISeq® 1500 (Illumina Inc. California, USA)
   xii) HiSeq® 2000 (Illumina Inc. California, USA)
   xiii) HiSeq® 2500 (Illumina Inc. California, USA)
   xiv) HiSeq® 4000 (Illumina Inc. California, USA)
   xv) HiSeq® X (Illumina Inc. California, USA)
   xvi) Ion Torrent PGM™ (Ion Torrent Systems, Inc., New Hampshire, USA)
   xvii) Ion Torrent Proton™ (Ion Torrent Systems, Inc., New Hampshire, USA)
   xviii) MGI Sequencer DNBSEQ-G400™ (MGI Tech, Shenzen, China)
   xix) MGI Sequencer DNBSEQ-G50™ (MGI Tech, Shenzen, China)

xx) MGI Sequencer DNBSEQ-T7™ (MGI Tech, Shenzen, China)
xxi) MiniSeq® (Illumina Inc. California, USA)
xxii) MiSeq® (Illumina Inc. California, USA)
xxiii) MiSeq® Dx (Illumina Inc. California, USA)
xxiv) MiSeq® FGx (Illumina Inc. California, USA)
xxv) NextSeq® (Illumina Inc. California, USA)
xxvi) NovaSeq® 6000 (Illumina Inc. California, USA)

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

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

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

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

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

h) Were the reads trimmed before bioinformatics analysis (please select one answer)? [**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

i) If reads were trimmed, which bioinformatics tool was used (please insert name and URL)?

j) 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/))?

k) 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)?

9) Questions regarding the analyses of sequences

9) Which species did you detect for EURGen-2023-01?
   i) Escherichia coli
   ii) Klebsiella pneumoniae
   iii) Acinetobacter baumannii
   iv) Pseudomonas aeruginosa
   v) No results submitted

10) Which species did you detect for EURGen-2023-02?
   i) Escherichia coli
   ii) Klebsiella pneumoniae
   iii) Acinetobacter baumannii
   iv) Pseudomonas aeruginosa
   v) No results submitted

11) Which species did you detect for EURGen-2023-03?
   i) Escherichia coli
   ii) Klebsiella pneumoniae
   iii) Acinetobacter baumannii
   iv) Pseudomonas aeruginosa
   v) No results submitted

12) Which species did you detect for EURGen-2023-04?
   i) Escherichia coli
   ii) Klebsiella pneumoniae
   iii) Acinetobacter baumannii
   iv) Pseudomonas aeruginosa
   v) No results submitted

13) For the detection of multi-locus sequence types (MLST), which methods did you apply (enter 'NA' if not applicable)?
   Please report information regarding:
   a) Pipeline type: local or web-based pipeline
   b) 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
   c) 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

d) Parameters of the software: default parameters or defined by you. If you used parameters defined by you, please specify them
e) If applicable, please specify the URL of the software and/or database used

14) For the detection of plasmids, which detection and/or typing methods did you apply (enter 'NA' if not applicable)?
Please report information regarding:
a) Pipeline type: local or web-based pipeline
b) 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
c) 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
d) 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.)
e) If applicable, please specify the URL of the software and/or database used

15) For the detection of antimicrobial resistance genes, which methods did you use (enter 'NA' if not applicable)?
Please report information regarding:
a) Pipeline type: local or web-based pipeline
b) 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
c) 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
d) 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.)
e) if applicable, please specify the URL of the software and/or database used

16) For the detection of chromosomal mutations mediating antimicrobial resistance, which methods did you apply (enter 'NA' if not applicable)?
Please report information regarding:

a) Pipeline type: local or web-based pipeline
b) 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
c) 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
d) 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.)
e) If applicable, please specify the URL of the software and/or database used

17) For the WGS-based prediction of antimicrobial resistance profiles, which methods did you apply (enter 'NA' if not applicable)?
Please report information regarding:
a) Pipeline type: local or web-based pipeline
b) 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
c) 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
d) 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.)
e) If applicable, please specify the URL of the software and/or database used

18) Questions regarding the analyses of sequencing data uploaded by EQA 2023 participants

a) Have the FASTQ files (raw, non-assembled sequence data, file names as indicated in the protocol) been uploaded to the ScienceData-folder for EURGen-2023-01?
i) Yes
ii) No
b) Have the FASTQ files (raw, non-assembled sequence data, file names as indicated in the protocol) been uploaded to the ScienceData-folder for EURGen-2023-02?
i) Yes
ii) No

c) Have the FASTQ files (raw, non-assembled sequence data, file names as indicated in the protocol) been uploaded to the ScienceData-folder for EURGen-2023-03?
   i) Yes
   ii) No
d) Have the FASTQ files (raw, non-assembled sequence data, file names as indicated in the protocol) been uploaded to the ScienceData-folder for EURGen-2023-04?
   i) Yes
   ii) No

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