PROTOCOL for the DTU Genomic Proficiency Test 2023

EU Reference Laboratory for Antimicrobial Resistance (EURL-AR)

October 2023

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1. Introduction

The DTU Genomic proficiency test (PT) 2023 is the fourth iteration of DTU Genomic PTs, and it is focused on whole genome sequencing (WGS) as well as *in silico* analyses, for bacterial typing and antimicrobial resistance (AMR) element identification. The main objective of the DTU Genomic PT is to test and compare technical and analytical skills of the participating laboratories within the areas of WGS and *in silico* analyses regarding bacterial typing and AMR element identification. Participation in the DTU Genomic PT 2023 will facilitate the development of reliable laboratory results to be used for monitoring and research purposes.

The DTU Genomic PT 2023 is coordinated by the National Food Institute, Technical University of Denmark (DTU), Denmark, and is funded by the Fleming Fund (SEQAFRICA Regional Grant) and the EU Commission, via the EU Reference Laboratory for Antimicrobial Resistance (EURL-AR). The Fleming Fund is a £265 million UK aid investment to tackle AMR in low- and middle-income countries around the world. The program is managed by the UK Department of Health and Social Care, in partnership with Mott MacDonald, the Fleming Fund Grants Management Agent. Via SEQFRICA, laboratories from the Africa Pathogen Genomics Initiative (Africa PGI) were also invited to register for participation.

The DTU Genomic PT 2023 focuses on the following three organisms, each represented by two strains (please note that signing up for each organism separately is allowed):

- *Escherichia coli* (×2 strains),
- *Salmonella enterica* (×2 strains) and
- *Staphylococcus aureus* (×2 strains)

Each strain of the above organisms is requested to be handled in two ways:

1a) **Live bacterial cultures** (referred to as "**BACT**"), for which DNA extraction, purification, library-preparation, WGS and *in silico* analyses are performed.

1b) **Pre-prepared, dried DNA** (referred to as "**DNA**"), for which WGS and *in silico* analyses are performed.

Note that item 1a and item 1b are parallel, *i.e.,* when signing up for 1a for one organism, the participation in 1b is also expected. Institutes/organizations that signed up to participate will receive the PT-material (cultures/pre-prepared DNA) according to the registered sign-up information.

Attention!

⇨ The DTU Genomic PT 2023 covers **single end or paired end sequence reads** (FASTQ files). Assessment of long read sequences (*e.g.*, ONT) is not offered as part of the present PT.

2. Shipping and receipt of test material

In October 2023, laboratories located in Europe (members of the EURL-AR network) and in Africa (SEQAFRICA partners and Africa PGI network members), will receive a parcel containing two *E. coli*, two *S. enterica* and two *S. aureus* strains in transport swabs, together with corresponding pre-prepared, dried DNA (contents of the parcel will correspond to the registered sign-up information). Bacterial transport swabs and dried DNA are shipped as UN3373, Biological substance category B. Information on the test material and the sample naming is presented in **Table 1**.

Attention!

⇨ **Please confirm receipt of the parcel through the confirmation form enclosed in the shipment.**

⇨ On arrival of the parcel to the laboratory, open the parcel to confirm that the contents are as listed in the cover letter.

***Table 1**. Test material and sample IDs for the DTU Genomic PT 2023.*

Organism	Strain ID	Test material type	Sample ID
E. coli	001	BACT	GENOMIC23-001-BACT
	001	DNA	GENOMIC23-001-DNA
	002	BACT	GENOMIC23-002-BACT
	002	DNA	GENOMIC23-002-DNA
S. enterica	003	BACT	GENOMIC23-003-BACT
	003	DNA	GENOMIC23-003-DNA
	004	BACT	GENOMIC23-004-BACT
	004	DNA	GENOMIC23-004-DNA
S. aureus	005	BACT	GENOMIC23-005-BACT
	005	DNA	GENOMIC23-005-DNA
	006	BACT	GENOMIC23-006-BACT
	006	DNA	GENOMIC23-006-DNA

3. Handling and storage of test material

3.1. Item 1a; Live bacterial cultures

Live bacterial cultures are shipped as transport swabs. Upon receipt, store the transport swabs at 5-25°C. The PT-organizers encourage participants to subculture and prepare the bacterial

cultures for storage in their strain collection (*e.g.,* in a -80°C freezer) within 48 hours from the receival of the parcel. The bacterial cultures supplied have been sequenced multiple times and the genomes have been closed.

3.2. Item 1b; pre-prepared, dried DNA

Each vial of the supplied pre-prepared, dried DNA contains at least 1 µg of DNA. The dried DNA sample can be stored in: (a) a dry storage cabinet at room temperature (15-25°C), or (b) a heat-sealed, moisture-barrier bag along with a silica gel desiccant pack, or (c) if sequencing of the samples is planned within the first 10 days of arrival of the shipment, the dried samples can be stored in 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. If you wish to store for more than 10 days, the samples can also be re-hydrated and stored, as described below.

The dried DNA samples should be re-hydrated in nuclease free water or aqueous buffer (recommended volume 100 µL). The DNA was dried in a vacuum centrifuge, and it is possible that material is present on the walls of the Eppendorf tube; therefore, to make sure all the DNA is dissolved, pipette up and down and let the water/buffer slide down all the sides of the Eppendorf tube. Incubate the tubes at room temperature for 15 minutes to allow complete hydration and then mix gently by pipetting, to re-suspend the sample.

The re-hydrated DNA can now be used directly in downstream applications or stored for up to one month at 4°C, or at room temperature, in closed tubes to prevent evaporation. For long-term storage, the re-hydrated DNA is recommended to be kept at a -80ºC freezer, if available, otherwise at a -20ºC freezer. Optionally, the quality 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.

4. Analysis of test material and result submission

The bacterial strains should be cultured on appropriate agar and at appropriate growth conditions, according to each laboratory's routine procedures. Following incubation and assessment of purity of the bacterial cultures, DNA extraction and WGS should be performed. For the pre-prepared DNA received, WGS should be performed. DNA extraction and WGS should be performed according to the laboratory's standard procedures. While handling both bacterial cultures and pre-prepared DNA (items 1a and 1b), register information about the methods applied via the **Test forms (Appendix 3)**.

Proceed to the *in silico* analyses of the obtained WGS data, which include:

- Multi-Locus Sequence Type (MLST)
- Plasmid replicon type
- AMR element identification (genes and chromosomal point mutations) and finally

- AMR phenotype prediction

Participants are requested to submit the raw sequencing data (FASTQ files) as well as results on the different bioinformatics analyses mentioned above. An overview of the requested data and results to be submitted is provided in **Table 2** .

***Table 2**. Overview of the requested data and results to be submitted for the DTU Genomic PT 2023.*

	Type of data/results
1	Raw sequencing data (FASTQ-files)
2	Method information
3	Multi-Locus Sequence Type (MLST)
4	Plasmid replicon type
5	AMR genes*
6	Chromosomal point mutations known to mediate AMR*
7	Predicted AMR phenotype*

** Only related to the antimicrobial compounds included in the DTU Genomic PT 2023, for each organism, see Table 3 and 4.*

Attention!

⇨ For the AMR component, please report genes, chromosomal point mutations and predicted phenotype **only for the antimicrobials included in the DTU Genomic PT 2023, for each organism, according to Table 3 and Table 4**.

***Table 3**. Antimicrobial agents included in the DTU Genomic PT 2023 for E. coli and S. enterica.*

Antimicrobial	Class
Amikacin	Aminoglycoside
Ampicillin	Beta-lactam
Azithromycin	Macrolide
Cefepime	Beta-lactam
Cefotaxime	Beta-lactam
Cefoxitin	Beta-lactam
Ceftazidime	Beta-lactam
Chloramphenicol	Amphenicol
Ciprofloxacin	Quinolone
Colistin	Polymyxin
Ertapenem	Beta-lactam
Gentamicin	Aminoglycoside
Imipenem	Beta-lactam
Meropenem	Beta-lactam
Nalidixic acid	Quinolone
Sulfamethoxazole	Folate pathway antagonist
Temocillin	Beta-lactam
Tetracycline	Tetracycline
Tigecycline	Tetracycline
Trimethoprim	Folate pathway antagonist

***Table 4**. Antimicrobial agents included in the DTU Genomic PT 2023 for S. aureus.*

Antimicrobial	Class
Cefoxitin	Beta-lactam
Chloramphenicol	Amphenicol
Ciprofloxacin	Quinolone
Clindamycin	Lincosamide
Erythromycin	Macrolide
Fusidate	Steroid antibacterial
Gentamicin	Aminoglycoside
Kanamycin	Aminoglycoside
Linezolid	Oxazolidinone
Mupirocin	Pseudomonic acid
Penicillin	Beta-lactam
Rifampin	Rifamycin
Streptomycin	Aminoglycoside
Sulfamethoxazole	Folate pathway antagonist
Tetracycline	Tetracycline
Tiamulin	Pleuromutilin
Trimethoprim	Folate pathway antagonist
Vancomycin	Glycopeptide

4.1. Submission of raw sequencing data

For submitting the raw sequencing data (FASTQ-files), the ScienceData platform will be used. Via the cover letter, each participant is informed about their individual link to the ScienceData platform. The cover letter is included as a hardcopy with the shipment of the PT material and is also forwarded by email to the PT contact persons, as a pdf document.

FASTQ-files related to item 1a and 1b should be uploaded to the ScienceData platform via the unique link for each laboratory mentioned above. For detailed information on how to upload your files in ScienceData, please consult Appendix 1. FASTQ files uploaded in your ScienceData folder by the submission deadline are considered for evaluation. Please name the FASTQ files according to the blue box below. When you have uploaded your files, **please check the size of the files in ScienceData to confirm that they correspond to the expected file sizes**. Pre-screening steps will be performed to check the sequence file format (FASTQ) and file name (as described in the blue box below). If the file format and file name are not compatible with the present submission guideline, the file will be excluded from further analysis.

Attention!

Before submitting the FASTQ-files in ScienceData, name them as following to match corresponding samples:

Material	Read	Sequence name in ScienceData folder
BACT	Read 1	2023-XX_GENOMIC23-00x-BACT_R1.fastq.gz
	Read 2	2023-XX_GENOMIC23-00x-BACT_R2.fastq.gz
DNA	Read 1	2023-XX_GENOMIC23-00x-DNA_R1.fastq.gz
	Read 2	2023-XX_GENOMIC23-00x-DNA_R2.fastq.gz

- ⇨ "**2023-XX**" refers to the **laboratory ID** you have been given as a participant, which is found at the top of your cover letter.
- ⇨ "GENOMIC23-00x-BACT" and "GENOMIC23-00x-DNA" refer to the sample name, *i.e.*, GENOMIC23-001-BACT to GENOMIC23-006-BACT for sequences from live cultures and GENOMIC23-001-DNA to GENOMIC23-006-DNA for sequences from the pre-prepared DNA.

4.2. How to submit results via the DTU webtool

The DTU Webtool is used for the submission of the method information as well as the results of all *in silico* analyses presented above. The webtool manual (Appendix 2) presents the procedure of result submission in detail and the PT organisers recommend that the laboratories read it carefully before submitting the results. The person(s) who will submit the results in the DTU Webtool are highly encouraged to have by their side the completed Test forms (Appendix 3).

The DTU Webtool can be accessed through this link: https://genomic-pt.dtu.dk. About login to the webtool, your **personal login ID** will be sent to you by email, along with details on how to generate a password, which will allow you to log in the webtool.

Attention!

- ⇨ Before finally submitting your results in the DTU Webtool, please ensure that you have filled in all the relevant fields, as **you can only 'finally submit' once!** "Final submit" blocks data entry.

4.2.1. Submission of method information

Details in relation to submission of method information via the DTU Webtool are described in Appendix 2. Test forms that present an overview for recording your results before you enter them in the DTU Webtool are available in Appendix 3. This is relevant for:

- Details in relation to received test material (mandatory),
- Details in relation to sequencing and analysis method (mandatory),
- Identification of MLST and plasmid replicon type (mandatory) and
- Identification of AMR genes, chromosomal mutations inducing AMR, upregulated AmpC (relevant for *E. coli*) and subsequently identification of the predicted AMR phenotype (mandatory).

4.2.2. Submission of bacterial typing data

MLST and plasmid replicon type are submitted via the DTU Webtool, in the relevant tabs. In the MLST tab, participants are asked to submit the allelic numbers for each of the seven chromosomal housekeeping genes included in the MLST scheme for each organism (**Table 5**). For *E. coli* please use the Achtman scheme[1] (*E. coli*#1, if using CGE MLST - https://cge.food.dtu.dk/services/MLST/). Participants should submit the sequence type (ST) for each sample. Enter "0" if the obtained result does not show a perfect match or if an allele cannot be detected.

***Table 5**. Alleles included in the MLST schemes relevant for the DTU Genomic PT 2023.*

	Allele 1	Allele 2	Allele 3	Allele 4	Allele 5	Allele 6	Allele 7
E. coli*	*adk*	*fumC*	*gyrB*	*icd*	*mdh*	*purA*	*recA*
S. enterica	*aroC*	*dnaN*	*hemD*	*hisD*	*purE*	*sucA*	*thrA*
S. aureus	*arcC*	*aroE*	*glpF*	*gmk*	*pta*	*tpi*	*yqiL*

*Achtman scheme (see text).

4.2.3. Submission of AMR data

The participants must carefully evaluate the AMR results before proceeding to submission via the DTU Webtool and a collaboration between a bioinformatician and a microbiologist with knowledge within AMR is highly recommended.

[1] Wirth T, Falush D, Lan R, Colles F, Mensa P, Wieler LH, Karch H, Reeves PR, Maiden MC, Ochman H, Achtman M. Sex and virulence in *Escherichia coli*: an evolutionary perspective. Mol Microbiol. 2006 Jun;60(5):1136-51. doi: 10.1111/j.1365-2958.2006.05172.x. PMID: 16689791; PMCID: PMC1557465.

AMR determinants (genes and chromosomal point mutations) and predicted AMR phenotype related to the antimicrobial compounds included in the DTU Genomic PT 2023 for each organism, according to Table 3 and Table 4, must be submitted.

Attention!

⇨ Submitted AMR genes, chromosomal point mutations known to mediate AMR or predicted AMR phenotype related to **other antimicrobial compounds than the ones listed in Table 3 and Table 4 for each organism are not part of the expected results and will be scored as incorrect results**.

Participants must carefully **evaluate multiple hits for variants of AMR genes that come from the same location in the genome**. In such case, the participants should select which variant to report based on the percent identity and percent coverage to the reference gene and report the best quality hit. Submission of several variants from the same position in the genome will be scored as an incorrect result.

4.3. Deadline for submission of results

Submission is successful after ticking off the 'final submit' in the DTU Webtool (see webtool manual, Appendix 2). Following 'final submit', the primary and secondary contact person receives and email with the submitted results as an attachment. Results must be submitted electronically **no later than 12 December 2023 at 16:00**. Immediately after this, the webtool will be closed for further edits and submission. Delayed submission of results will not be accepted.

5. Evaluation of the submitted results

Submitted results are evaluated in two parts: a) the QC of the raw sequencing data and the assemblies and b) the *in silico* analyses. The participating laboratories will receive an email from the DTU Genomic PT 2023 organizer when the evaluation is completed. **The evaluation will not indicate pass/fail.** In the following parts details on the evaluation of the two parts are provided. The submitted information on the methods applied are not evaluated but are used as background information.

5.1. Evaluation of the raw sequence data

For both bacterial cultures and pre-prepared DNA (items 1a and 1b), the evaluation will be based on the submitted sequence data (FASTQ-files) which will be assembled using SPAdes

(http://bioinf.spbau.ru/spades) and run through a QC pipeline by the PT organizers. The output from the QC analysis is collected in two tables: a summarizing scoring table and an elaborating QC parameter table. The QC parameter table contains the specific values from the QC analysis, including (but not limited to) those used for scoring. The scoring table sums up the general performance of each sample based on the following criteria:

- Average coverage,
- MLST,
- Q-score of R1,
- Q-score of R2,
- Proportion of correct cgMLST genes identified,
- Number of contigs,
- N50,
- Genomic coverage of minimal depth 10x,
- Proportion of reads mapping to reference genome, and
- Size of the assembled genome compared to the reference genome.

The evaluation will not indicate pass/fail and 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.

5.2. Evaluation of *in silico* analyses

For the *in silico* analyses (MLST, plasmid replicon type, AMR part) an individual evaluation report will be generated for each laboratory. Upon login to the DTU Webtool, clicking on 'Download report' will give access to the evaluation report presenting obtained results, expected results and scores. The evaluation will not indicate pass/fail.

In the DTU Genomic PT 2023 the reported results for each *in silico* analysis are evaluated individually and every submitted result will receive a score of "1" or "0", according to **Table 6**. When a submitted result is on the list of expected results, a score of "1" is achieved, whereas a mismatch (obtained result is not expected) is scored with a "0". Expected results not submitted as obtained results are presented in the evaluation report and are scored with a "0".

***Table 6**. Scoring of in silico analysis results in the DTU Genomic PT 2023.*

Scenario	Obtained score
Submission of expected data	**1**
Submission of unexpected data	**0**
Not submitting expected data	**0**

Attention!

⇨ In the DTU Genomic PT 2023, predicted AMR phenotype to each antimicrobial compound listed in Table 3 and Table 4, for each organism, will be scored **individually and not as a profile, as done in the previous iterations of the DTU Genomic PT.**

6. Analysis and publication of results

A guidance document will be sent to the laboratories presenting a summary of the results of the DTU Genomic PT 2023 and scientific background for the evaluation. Possibly, the results will be subsequently published in a peer-reviewed publication. Authors and co-authors of the publications will be those who have contributed to the preparation and execution of the PT. Due to the anonymity of the results, the individual participating laboratories will not be acknowledged in the publications.

Individual results will be anonymized using laboratory codes which are confidential and known only to the individual laboratory and the PT-organizers. For laboratories related to the Fleming Fund grant, SEQAFRICA, the complete list of laboratory codes is known to the project management team, Mott MacDonald and the Fleming Fund. For laboratories participating as part of the EURL-AR network, the complete list of laboratory codes is known to the EU Commission.

7. CONTACT

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

DTU Genomic PT 2023 Coordinator:

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Appendix 1

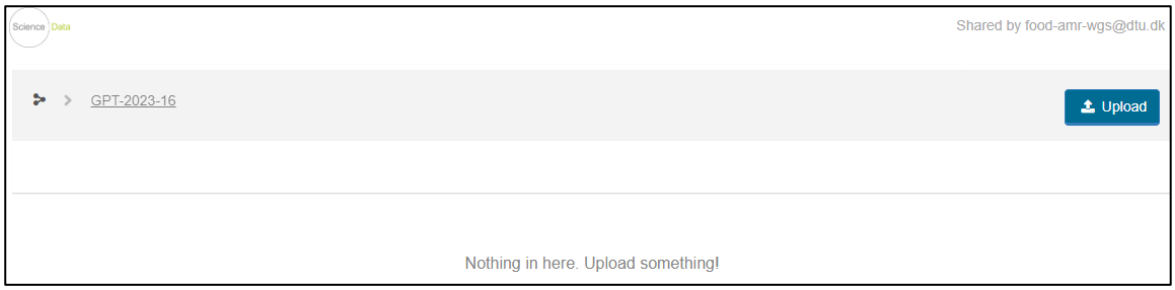
Using ScienceData to transfer FASTQ files

ScienceData is a cloud-based storage data platform built and operated by the Technical University of Denmark (DTU). Please access ScienceData using the link provided to you **in the cover letter**. The cover letter can be found in the DTU Genomic PT parcel received by the laboratories (as a hardcopy), as well as attached to an email sent to the PT contact persons (pdf file).

How to upload files to the ScienceData platform:

1. Confirm that the FASTQ files are correctly named, according to the DTU Genomic PT 2023 protocol, section 4.1 “Submission of raw sequencing data”.

2. Click on the link provided by the proficiency test organizer and see the following image (example):



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

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

Attention!

Please note that in the ScienceData folders **it is not possible to delete files**.

Therefore, in case you wish to delete uploaded files, please contact the DTU Genomic PT 2023 Coordinator, Susanne Karlsmose Pedersen (suska@food.dtu.dk).

Appendix 2

DTU Genomic PT webtool guideline

1. Browser requirements

IMPORTANT: The system works with the following browsers

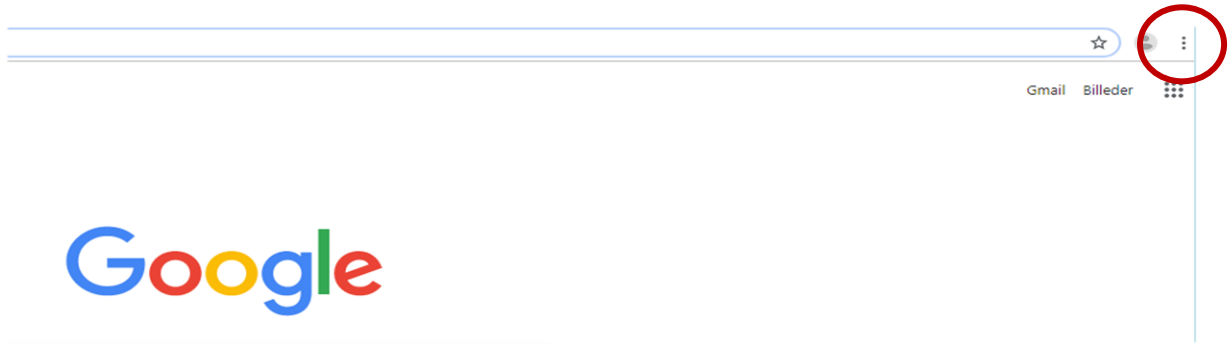
Browser	Oldest supported version*
Google Chrome	44.0
Firefox	39.0

* latest version is recommended.

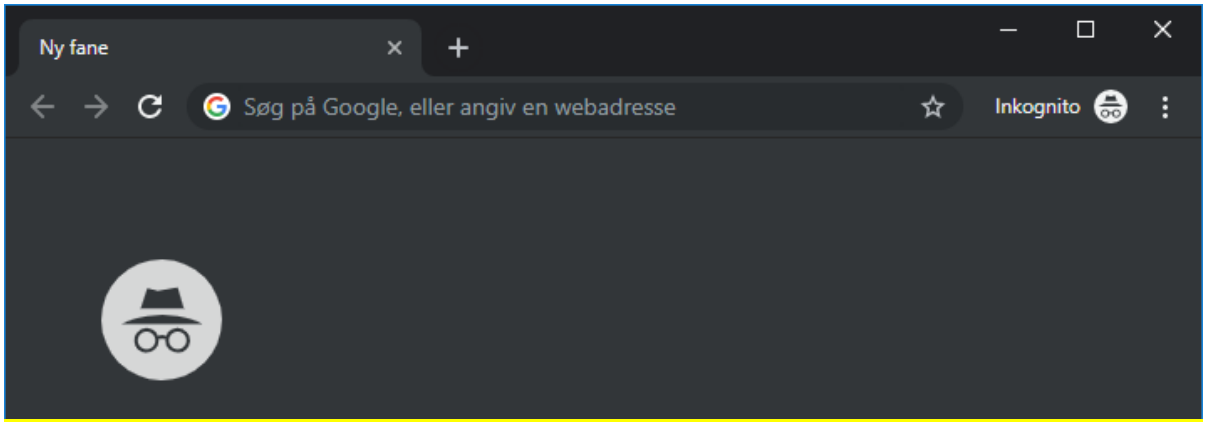
2. Access the webtool

IMPORTANT: To access the webtool, you must use an **incognito window**. NOTE: Should you have issues with requesting an incognito window, please contact the PT Coordinator (suska@food.dtu.dk) directly.

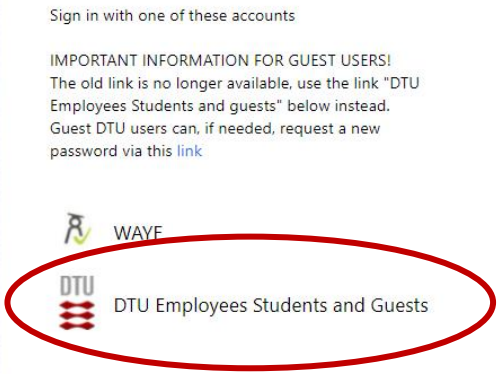
Open a browser window, click on the three dots (see red circle below) and select: 'New incognito window' (relevant when using Google chrome).



Continue in the black window that looks like this (relevant when using Google chrome):



Access the webtool using this address: https://genomic-pt.dtu.dk.



Select: DTU Employees Students and Guests.

Login to the system by using the **username** and **password** sent to you by e-mail for participation in PTs arranged by DTU Food.

3. How to create the password before granted get access the EQA webtool

Mandatory

Go to https://guest.dtu.dk/Sites/GuestLogin/RetrievePassword.aspx

After clicking the link, you can follow the below steps to reset your password.

Step 1

1. Enter your Username or the Email associated with your profile.

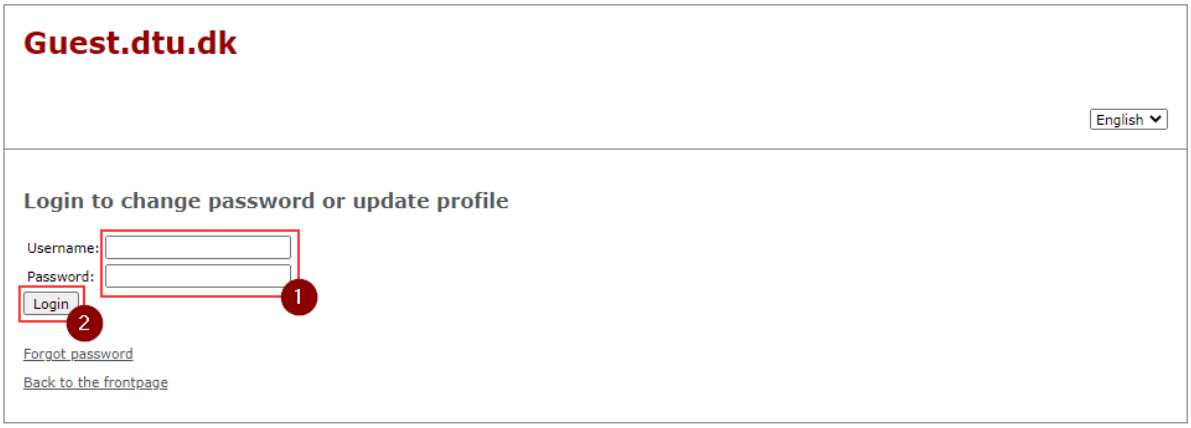
2. Click on Email, an email will shortly be sent to the email address associated with the profile.

Optional

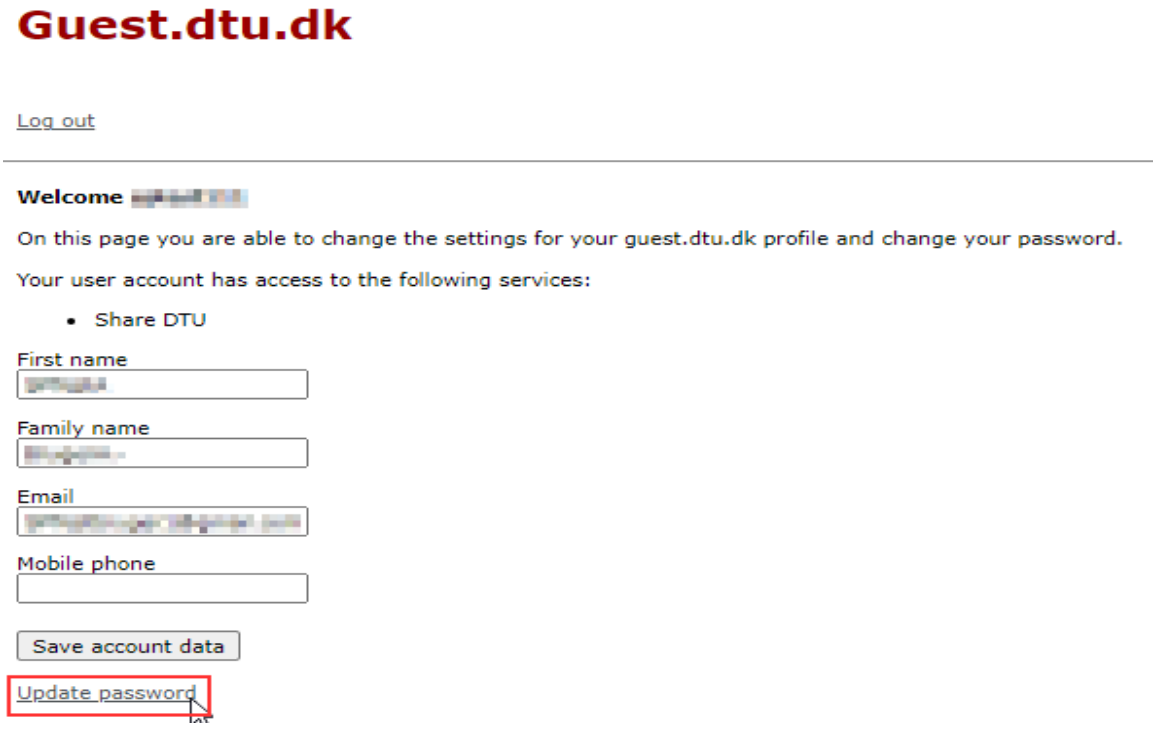
Here you can change your password after resetting of the password.
Go to https://guest.dtu.dk/Sites/GuestLogin/Default.aspx
After clicking the link, you can follow the below steps to change your password.

Step 1
1. Enter your username and password.

2. Click on (Login).

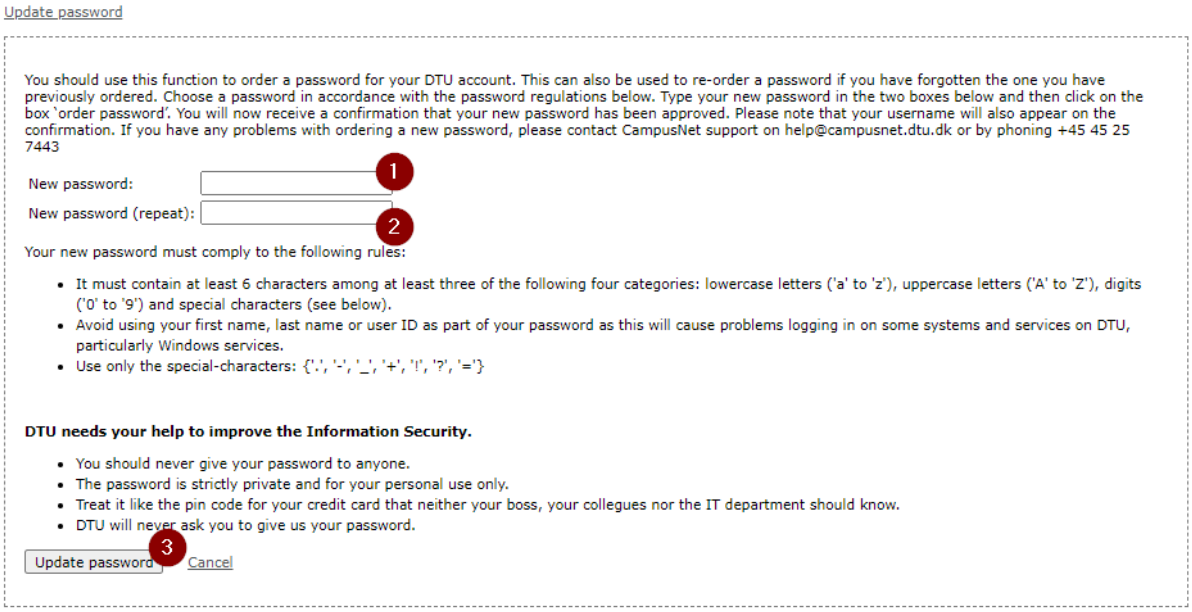


Step 2
Once logged in, click on (Update password).



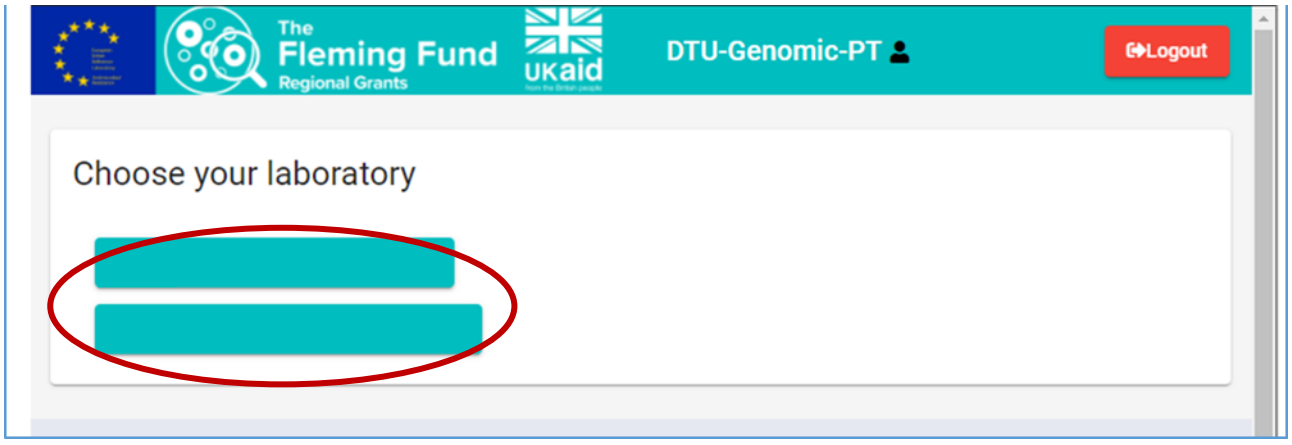
Step 3

1. Under (Update password) please fill out the field for your news password
    a. The password must contain at least 12 and at most 50 characters (a-Z, 0-9, {-,.,_,=,?,!,+})
    b. Avoid using your first name, last name or user ID as part of your password as this will cause problems when logging in on some systems and services on DTU, particularly Windows services
    c. You are not allowed to use number sequences such as e.g. "123" or "654"
    d. You are not allowed to repeat the same character/number three or more times in a row, e.g. "aaa" or "000"
2. Write the same password again in the (repeat) field so that the texts in the two fields match
3. Click on the (Update password) button to save the password change



Please note that it can take up to 15 minutes before the password change is in the system.

After sign in you will reach the *Proficiency Test Overview* page

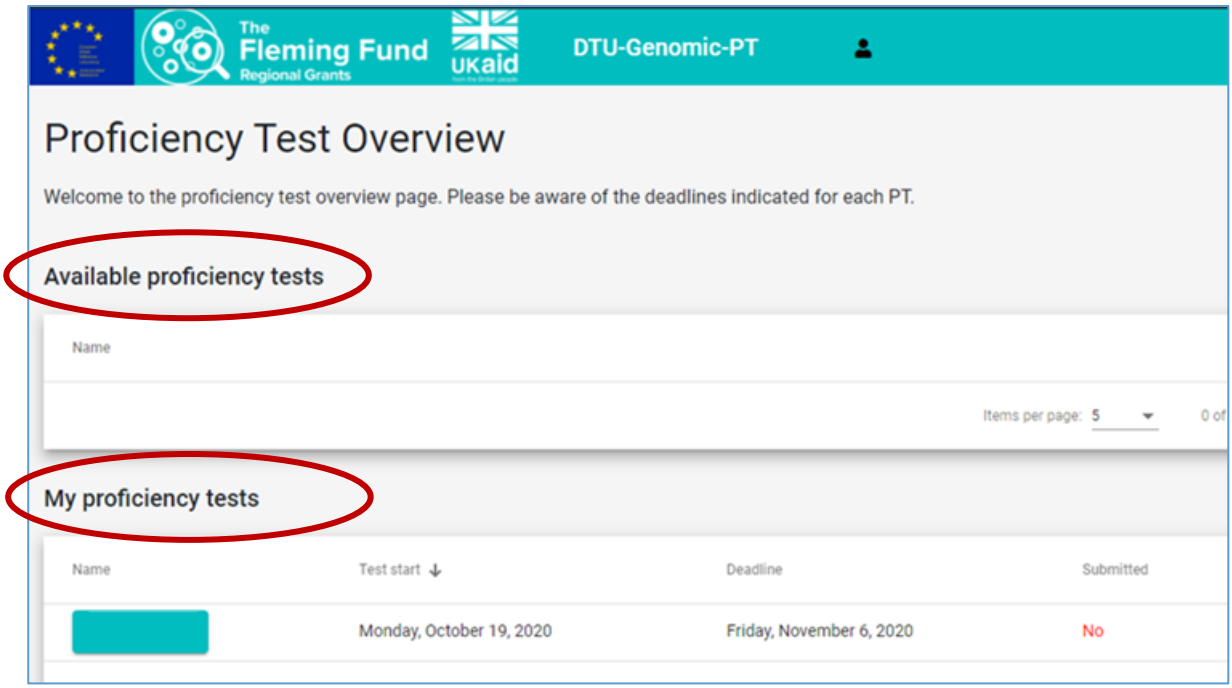


If you are connected to more than one specific laboratory, you will need to select the specific laboratory that you intend to submit results for.

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.

4. Signup or deselect

Under 'Available proficiency tests', sign-up to the relevant proficiency test. Proficiency tests you signed up to will be listed under 'My proficiency tests'.



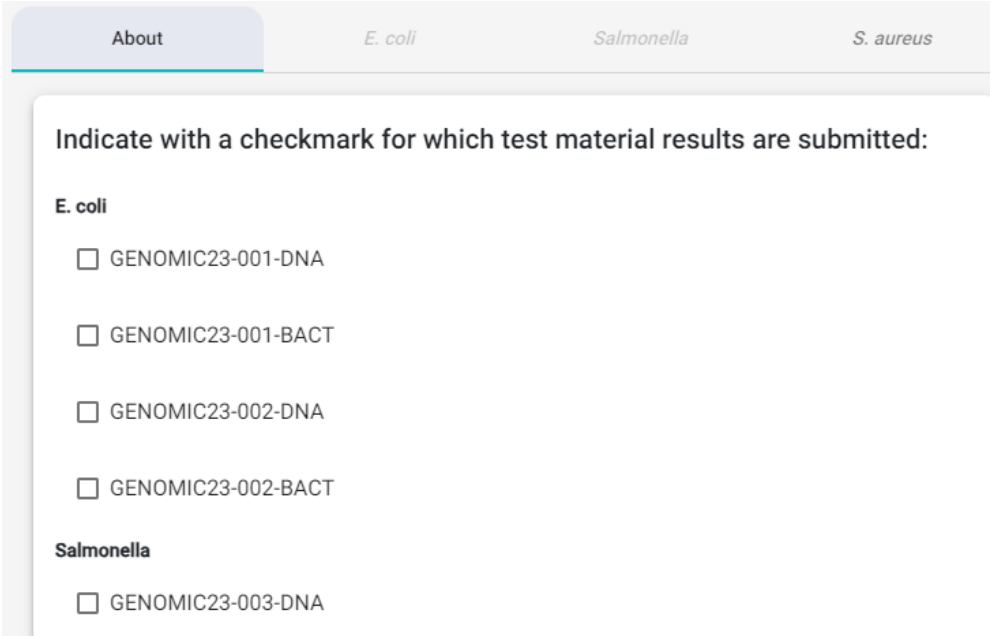
5. Navigate in the webtool

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

I. **'About' tab**. Enter data regarding the receipt and the storage PT test material. In this tab, with a checkmark, select the test material for which you wish to submit results, i.e. under *E. coli*, *Salmonella* and *S. aureus* select each relevant code and material.
II. **'Method' tab**. Enter data regarding processing of the bacterial cultures, the DNA received, DNA purification, sequencing and sequence analysis.
III. **'AMR' tab**. Enter data regarding AMR genes, chromosomal mutations, and predicted phenotype.
IV. **'MLST' tab**. Enter data regarding sequence type and alleles.
V. **'Plasmid replicon' tab.** Enter data regarding the plasmid replicon type(s) identified in each strain.
VI. **Save data**

I) 'About' tab

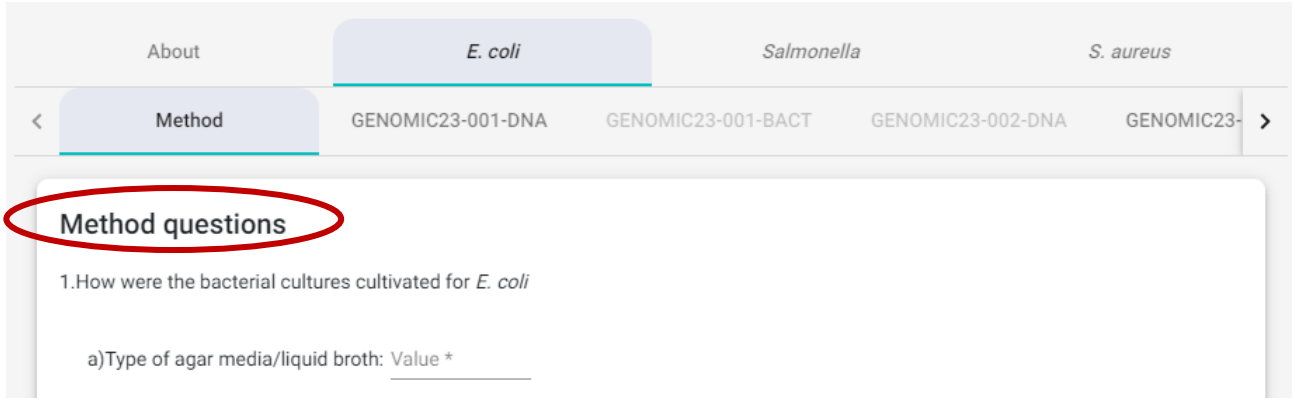
Respond to the general questions (an asterisk (*) indicates that a response is mandatory to complete the proficiency test) and select the test material that results will be submitted for. If a test material is selected under the 'About' tab, remaining tabs related to this test material will be activated and allows for access to enter results.



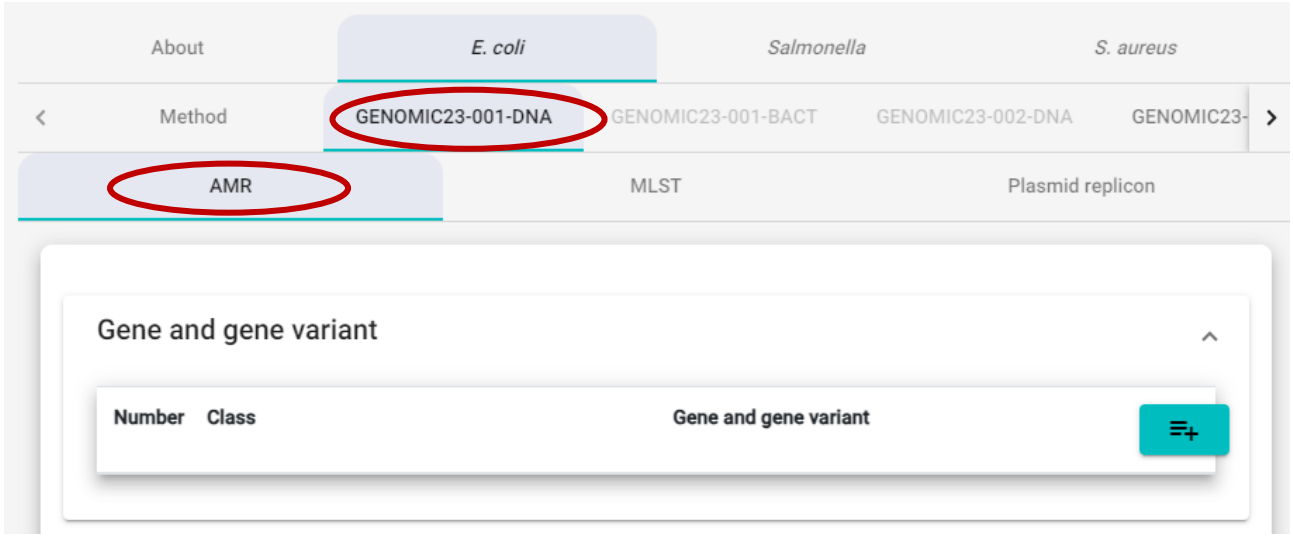
II) 'Method' tab

For each of the selected test material, respond to the questions in the 'Method' tabs. An asterisk (*) indicates that a response is mandatory to complete the proficiency test. Initially, there are some introductory questions regarding growth conditions and DNA extractions methods. Hereafter, the questions are divided into the following sections:

- **For the BACTERIAL CULTURES received**
- **For the DNA received**
- **SEQUENCING**
- **ANALYSIS of sequences**
- **SUBMITTED datafiles**



Select the tab related to one of the test strains (e.g. GENOMIC23-001-DNA). This opens for access to additional tabs.



III) 'AMR' tab:

Under the AMR-tab, results related to 1) identification of antimicrobial resistance genes, 2) chromosomal mutations inducing antimicrobial resistance, 3) upregulated AmpC (relevant for *E. coli*) and 4) identification of the predicted phenotype of the culture/pre-prepared DNA are uploaded.

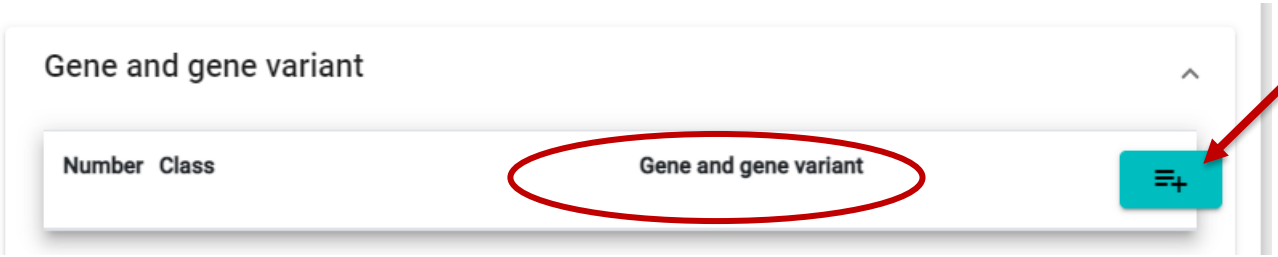
The submission of AMR results is mandatory and entered results will be evaluated.

Attention!

⇨ For the AMR component, please report genes, chromosomal point mutations and predicted phenotype **only for the antimicrobials included in the DTU Genomic PT 2023, for each organism, according to Table 3 and Table 4 in the DTU Genomic PT 2023 protocol**.

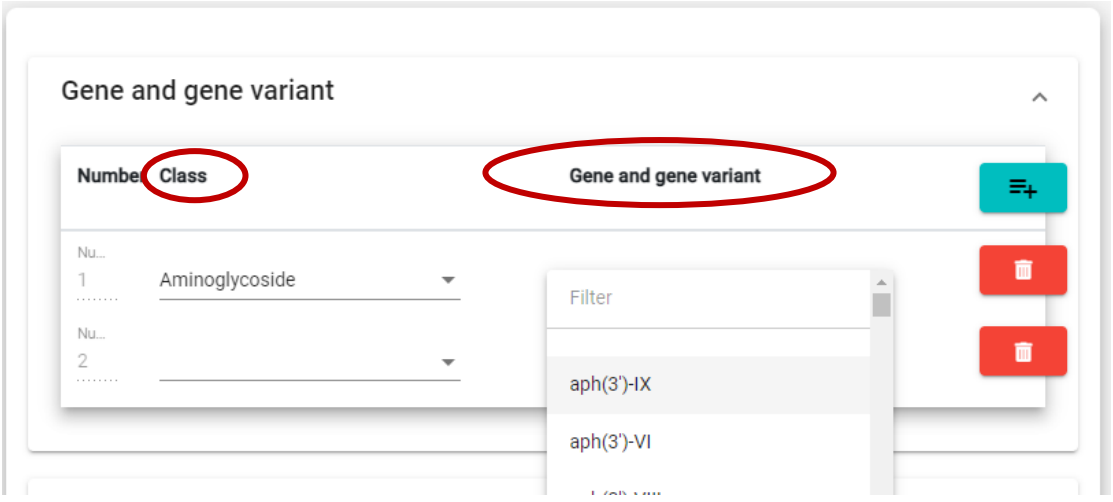
⇨ Submitted AMR genes, chromosomal point mutations known to mediate AMR or predicted AMR phenotype related to **other antimicrobial compounds than the ones listed in Table 3 and Table 4 in the DTU Genomic PT 2023 protocol, for each organism, are not part of the expected results and will be scored as incorrect results**.

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



In the drop-down menu under 'Class', select the antimicrobial class of the gene and gene variant you wish to report. Hereafter, from the drop-down 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 the gene variant name or parts of the gene variant name in the 'Filter'-field.

Add more lines by clicking on the '+' once again to proceed with submitting further genes and gene variants.

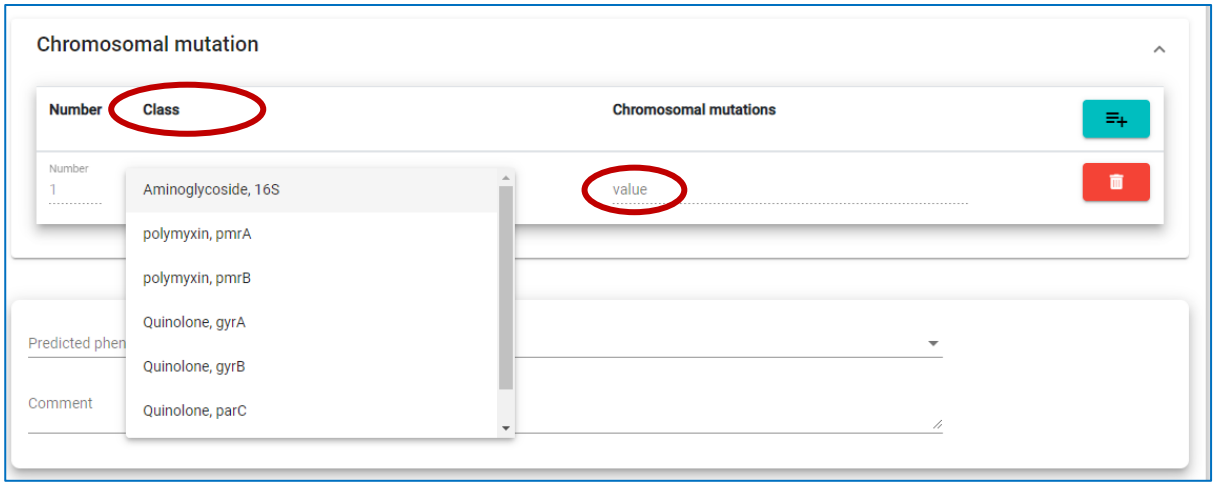


Some antimicrobial resistance genes are associated with resistance to more than one class of antimicrobials. To select these genes, select a 'Class' containing multiple classes and subsequently select the specific gene and gene variant.

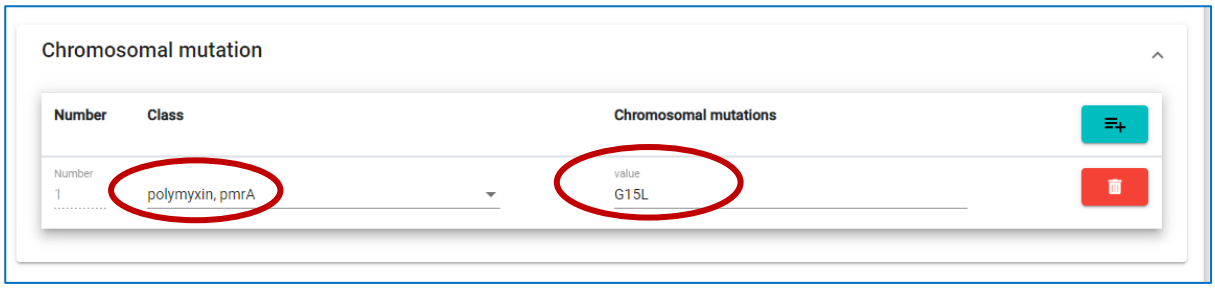
Ensure that no empty lines are saved for evaluation by clicking on the bin if you by mistake added one too many.

'Chromosomal mutations': In the drop-down menu under 'Class', select the specific class of antimicrobial followed by the mutated gene you wish to report a chromosomal mutation for. 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, or nucleotide for 16S or 23S sequences)
4) Please report the chromosomal mutations at a **protein level** (not DNA).

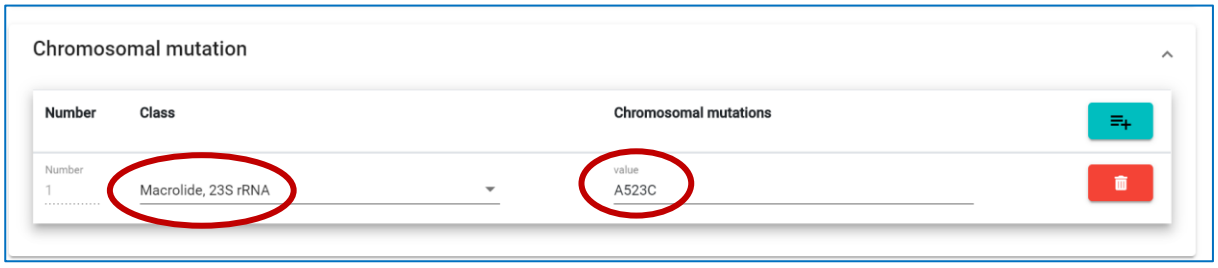


Example 1: Reporting a mutation in the *pmr*A gene which has changed the *amino acid* glycine (G) to Leucine (L) at position 15. This results in resistance to colistin that belongs to the polymyxin class of antimicrobials. Therefore, from the drop-down list under 'Class', select the 'polymyxin, *pmr*A'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

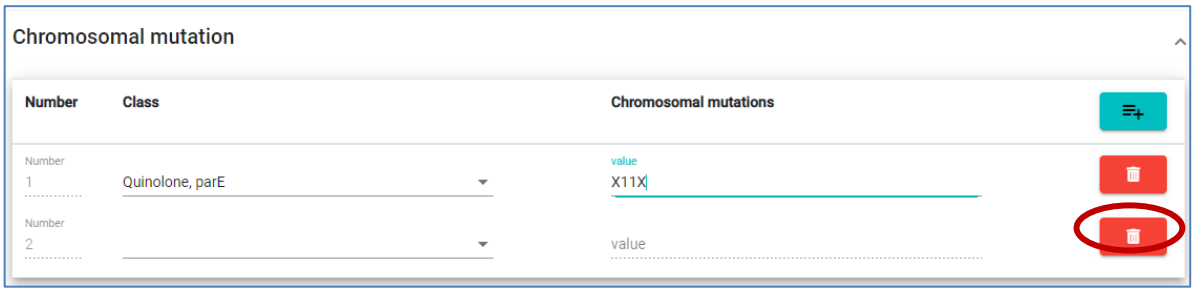


Example 2: If the mutation is in a 16S or a 23S rRNA gene please select the class of antimicrobial and associate gene (e.g. Macrolide, 23S rRNA) from the drop down menu. Hereafter, in the 'value', write the letter of the

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. A523C). Same principle as for the amino acids.

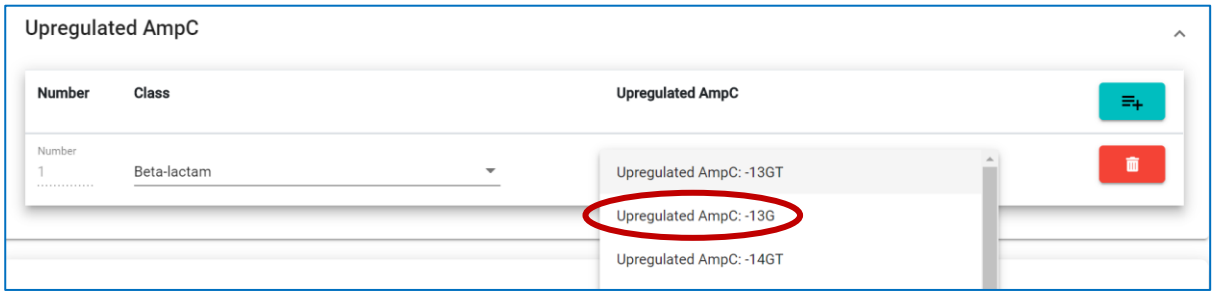


Ensure that no empty lines are saved for evaluation by clicking on the bin if you by mistake added one too many.

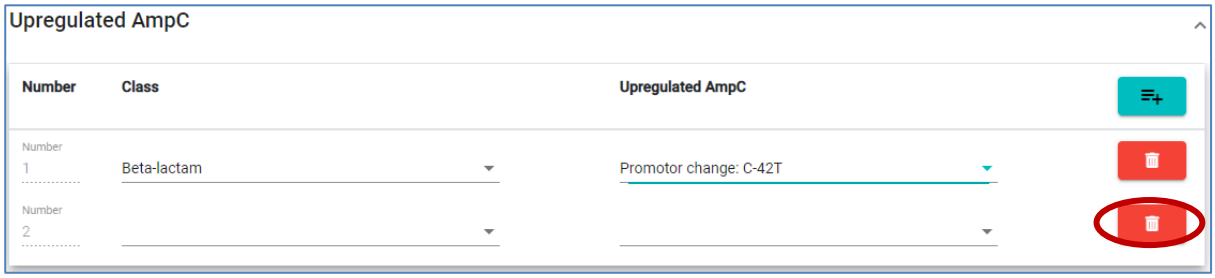


'Upregulated AmpC': For *E. coli,* upregulated AmpC resistance can be reported by selecting the 'Upregulated AmpC' option under the 'AMR' tab. For the 'Upregulated AmpC' option, select 'Beta-lactam' under 'Class' and hereafter, from the drop down menu under 'Upregulated AmpC', select the specific mutations 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).

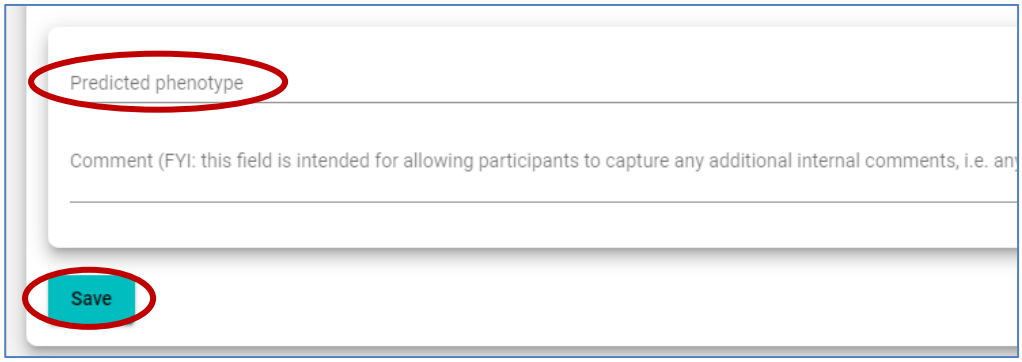


Ensure that no empty lines are saved for evaluation by clicking on the bin if you by mistake added one too many.



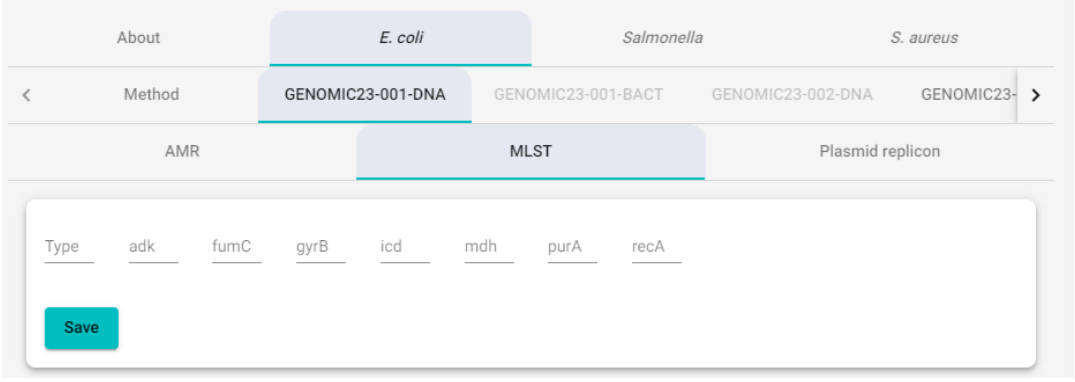
'Predicted phenotype': Click on 'Predicted phenotype' to select the antimicrobials that the sequence analysis indicates resistance to in the bacterial test strain. Note that in relation to predicted phenotype, antimicrobial resistance towards a limited number of antimicrobials is considered in this PT. Antimicrobials represented in the DTU Genomic PT are presented in the protocol, Tables 3 and 4.

'Comment': Any comments related to the submission of the results are welcome. You may for example indicate mutations that have unknown effect on antimicrobial resistance. Note, however, that these results will *not* be further evaluated.



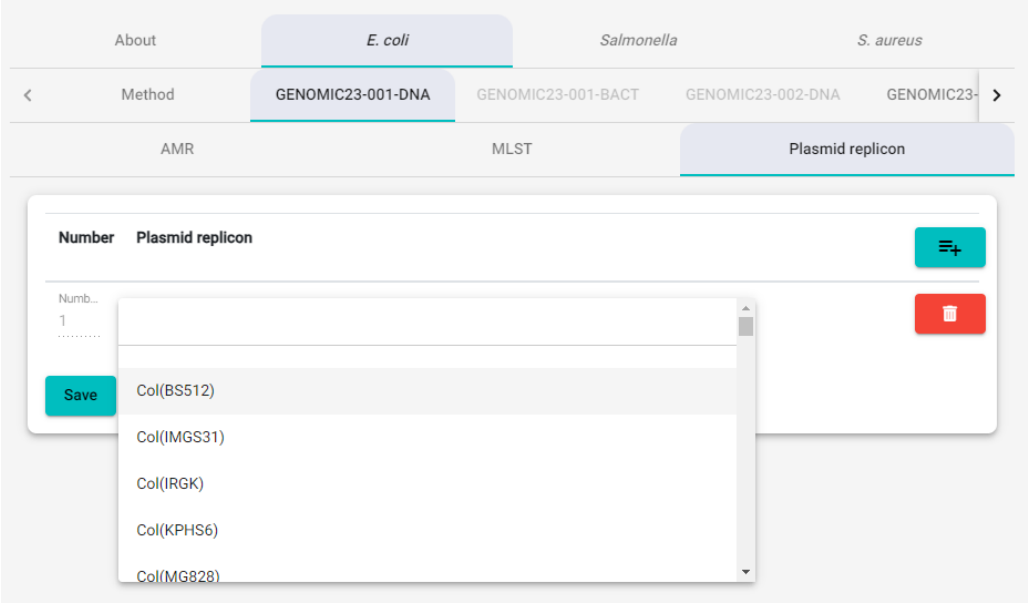
IV) 'MLST' tab

Enter data regarding MLST results (MLST type and corresponding allele numbers). Enter "0" if the obtained result does not show a perfect match or if an allele cannot be detected.



V) 'Plasmid replicon' tab

Select the plasmid replicon type from the drop-down list. Click on the "+" button on the right to add more lines. There should be a separate line for each submitted replicon. Entries can be deleted by clicking on the red bin button.



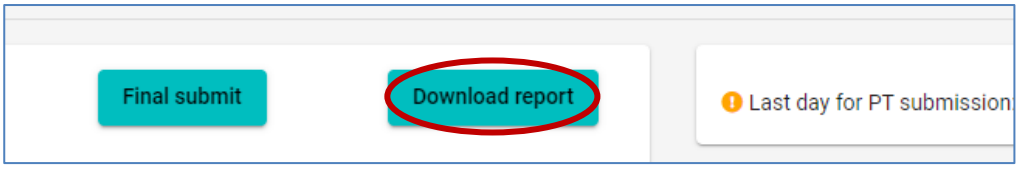
VI) Save data

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

Moreover, data are saved when you navigate to another tab.

6. 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 PT.



Before you have finally submitted your results (and before deadline), the database allows you to return to any testform and revise values.

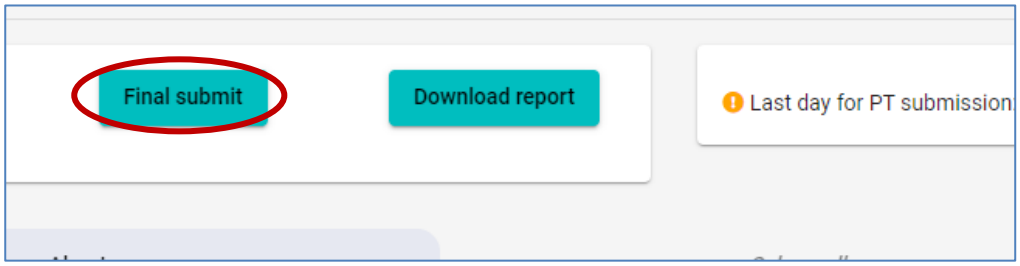
7. Submit data

For all organisms of the DTU Genomic PT 2023, all uploaded data are submitted in one go.

When all information and data have been entered and revised for i) the method, and, ii) all three test organisms, please indicate with a checkmark your acceptance that the uploaded data are ready for submission.

IMPORTANT! You will **NOT** be able to edit your data after final submission.

Click on 'Final Submit'



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

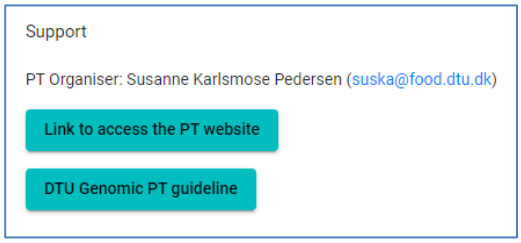
8. Evaluation report

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

9. Support

Should you need support in using the webtool, please do not hesitate to contact the PT 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 PT organizer. Find also link to the DTU Genomic PT website (to access the relevant PT protocol) as well as access to the current webtool manual.



Appendix 3

Test Forms – overview of method information and results of *in silico* analyses to be submitted via the DTU Webtool

With these test forms we invite you to obtain an overview of the data that must/may be submitted to the webtool.

You will find questions in relation to:

1. Sample storage and preprocessing
2. Bacterial Culture; DNA Isolation, Handling and Processing
3. Received DNA; Handling and Processing
4. Sequencing
5. Analysis of sequences; MLST, Plasmid replicon type and antimicrobial resistance
6. Submitted datafiles

Below, you find questions divided between sections entitled 'About' and 'Method'.

In the webtool, the 'About' section has one tab covering all three organisms, whereas the 'Method'-section has a tab for each organism. Therefore, even if in the below document you see the questions collected for all species, you will find *three 'Method' tabs in the webtool* and you would need to upload information in each of these, *i.e.,* once per organism that you wish to submit results for.

Note: An asterisk (*) indicates a question that requires an answer.

ABOUT

Sample storage and preprocessing

1) Date parcel with PT-material was received*:
   [DD/MM/YYYY]

2) Storage conditions of the bacterial cultures in the time between reception and processing (please select one answer)*:
   - -80˚C
   - -20˚C
   - 4˚C
   - Room temperature
   - No storage time
   - Other

   If other, please define:

3) Storage conditions of the DNA in the time between reception and processing (please select one answer)*:
   - -80˚C

- -20˚C
- 4˚C
- Room temperature
- No storage time
- Other

If other, please define

METHOD

Note that response to the questions in the 'Method' section below will be requested for each of the organisms that you wish to submit results for.

1. How were the bacterial cultures cultivated*:
   a) Type of agar media/liquid broth:
      E. coli:
      S. enterica:
      S. aureus:

   b) Incubation time (hours):
      E. coli:
      S. enterica:
      S. aureus:

   c) Incubation temperature (˚C):
      E. coli:
      S. enterica:
      S. aureus:

2. Please fill out information about the applied DNA extraction procedure and indicate any modifications from the kit used.
   a) If manual extraction; kit used, full name:
      E. coli:
      S. enterica:
      S. aureus:

   b) If manual extraction; catalogue number of kit:
      E. coli:
      S. enterica:
      S. aureus:

   c) If manual extraction, modifications to kit protocol:
      E. coli:
      S. enterica:
      S. aureus:

   d) If automatic extraction; robot used:
      E. coli:
      S. enterica:
      S. aureus:

e) If automatic extraction; specific protocol:
E. coli:
S. enterica:
S. aureus:

f) If automatic extraction; modifications to protocol:
E. coli:
S. enterica:
S. aureus:

For BACTERIAL CULTURES received

3. For bacterial cultures, how was the DNA concentration (ng/µl) prior to library preparation measured (please select one answer)*
   - Qubit® (Invitrogen™/Thermo Fisher Scientific) NanodropTM (Thermo Fisher Scientific) BioanalyzerTM (Agilent Technologies)
   - DNA concentration not measured
   - Other

   If other, please define:

4. Measurement of DNA concentration (ng/µl) for each test strain (bacterial cultures received)*
   For *E. coli*, GENOMIC23-001-BACT:
   For *E. coli*, GENOMIC23-002-BACT:
   For *S. enterica*, GENOMIC23-003-BACT:
   For *S. enterica*, GENOMIC23-004-BACT:
   For *S. aureus*, GENOMIC23-005-BACT:
   For *S. aureus*, GENOMIC23-006-BACT:

5. Please provide the total DNA amount (microgram) for each test strain (bacterial cultures received) (not mandatory)
   For *E. coli*, GENOMIC23-001-BACT:
   For *E. coli*, GENOMIC23-002-BACT:
   For *S. enterica*, GENOMIC23-003-BACT:
   For *S. enterica*, GENOMIC23-004-BACT:
   For *S. aureus*, GENOMIC23-005-BACT:
   For *S. aureus*, GENOMIC23-006-BACT:

6. For bacterial cultures, how was the DNA quality (*e.g.* 260/280 ratio and/or 260/230 ratio) prior to library preparation measured (please select one answer) (not mandatory)
   - Bioanalyser
   - Nanodrop
   - DNA quality not measured
   - Other

   If other, please define

7. If relevant, following your response to the previous question, measurement of DNA quality (*e.g.* Bioanalyser, 260/280 ratio, other) for each test strain (bacterial cultures received)
   For *E. coli*, GENOMIC23-001-BACT:
   For *E. coli*, GENOMIC23-002-BACT:
   For *S. enterica*, GENOMIC23-003-BACT:
   For *S. enterica*, GENOMIC23-004-BACT:
   For *S. aureus*, GENOMIC23-005-BACT:
   For *S. aureus*, GENOMIC23-006-BACT:

8. If relevant, measurement of DNA quality (260/230 ratio) for each test strain (bacterial cultures received) (not mandatory)
   For *E. coli*, GENOMIC23-001-BACT:
   For *E. coli*, GENOMIC23-002-BACT:
   For *S. enterica*, GENOMIC23-003-BACT:
   For *S. enterica*, GENOMIC23-004-BACT:
   For *S. aureus*, GENOMIC23-005-BACT:
   For *S. aureus*, GENOMIC23-006-BACT:

For DNA received

9. For DNA received, how was the DNA concentration (ng/µl) prior to library preparation measured (please select one answer)*
   - Qubit® (Invitrogen™/Thermo Fisher Scientific)
   - NanodropTM (Thermo Fisher Scientific)
   - BioanalyzerTM (Agilent Technologies)
   - DNA concentration not measured
   - Other

   If other, please define

10. Measurement of the DNA concentration (ng/µl) for each test strain (for the DNA received) *
   For *E. coli*, GENOMIC23-001-DNA:
   For *E. coli*, GENOMIC23-002-DNA:
   For *S. enterica*, GENOMIC23-003-DNA:
   For *S. enterica*, GENOMIC23-004-DNA:
   For *S. aureus*, GENOMIC23-005-DNA:
   For *S. aureus*, GENOMIC23-006-DNA:

11. Measurement of the total DNA amount (microgram) for each test strain (for the DNA received) (not mandatory)
   For *E. coli*, GENOMIC23-001-DNA:
   For *E. coli*, GENOMIC23-002-DNA:
   For *S. enterica*, GENOMIC23-003-DNA:
   For *S. enterica*, GENOMIC23-004-DNA:
   For *S. aureus*, GENOMIC23-005-DNA:
   For *S. aureus*, GENOMIC23-006-DNA:

12. For DNA received, how was the DNA quality (*e.g.* 260/280 ratio and/or 260/230 ratio) prior to library preparation measured (please select one answer) (not mandatory)
    - Bioanalyser
    - Nanodrop
    - DNA quality not measured
    - Other

    If other, please define

13. If relevant, following your response to the previous question, measurement of DNA quality (*e.g.* Bioanalyser, 260/280 ratio, other) for each test strain (for DNA received)
    For *E. coli*, GENOMIC23-001-DNA:
    For *E. coli*, GENOMIC23-002-DNA:
    For *S. enterica*, GENOMIC23-003-DNA:
    For *S. enterica*, GENOMIC23-004-DNA:
    For *S. aureus*, GENOMIC23-005-DNA:
    For *S. aureus*, GENOMIC23-006-DNA:

14. If relevant, measurement of DNA quality (260/230 ratio) for each test strain (for DNA received) (not mandatory)
    For *E. coli*, GENOMIC23-001-DNA:
    For *E. coli*, GENOMIC23-002-DNA:
    For *S. enterica*, GENOMIC23-003-DNA:
    For *S. enterica*, GENOMIC23-004-DNA:
    For *S. aureus*, GENOMIC23-005-DNA:
    For *S. aureus*, GENOMIC23-006-DNA:

15. Did you perform quality check to verify the quality of the DNA on a gel (yes/no) (see description in the protocol of this optional check)
    For *E. coli*, GENOMIC23-001-DNA:
    For *E. coli*, GENOMIC23-002-DNA:
    For *S. enterica*, GENOMIC23-003-DNA:
    For *S. enterica*, GENOMIC23-004-DNA:
    For *S. aureus*, GENOMIC23-005-DNA:
    For *S. aureus*, GENOMIC23-006-DNA:

SEQUENCING

16. Which protocol was used to prepare the sample library for sequencing? For commercial kits please provide the full kit name and catalogue number. For noncommercial 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 relevant)*:

    a) For commercial kits; full kit name:
    b) For commercial kits; catalogue number:
    c) For noncommercial kits; citation for the protocol:

d) For noncommercial kits; summary of the protocol:
e) Deviations from the kit or cited protocol

17. Please indicate the sequencing platform you used in the proficiency test (please select one answer)*:
- ABI SOLiD™ (Thermofisher Scientific, Massachusetts, USA)
- Genome Analyzer Iix (Illumina Inc. California, USA)Genome Sequencer FLX™ System (454) (Roche Holding AG, Basel, Switzerland)
- Genome Sequencer FLX+™ System (454) (Roche Holding AG, Basel, Switzerland)
- Genome Sequencer Junior™ System (454) (Roche Holding AG, Basel, Switzerland)
- HiScan™ SQ System (Illumina Inc. California, USA)
- HiSeq® 1000 (Illumina Inc. California, USA)
- HISeq® 1500 (Illumina Inc. California, USA)
- HiSeq® 2000 (Illumina Inc. California, USA)
- HiSeq® 2500 (Illumina Inc. California, USA)
- HiSeq® 4000 (Illumina Inc. California, USA)
- HiSeq® X (Illumina Inc. California, USA)
- Ion Torrent PGM™ (Ion Torrent Systems, Inc., New Hampshire, USA)
- Ion Torrent Proton™ (Ion Torrent Systems, Inc., New Hampshire, USA)
- MGI Sequencer DNBSEQ-G400™ (MGI Tech, Shenzen, China)
- MGI Sequencer DNBSEQ-G50™ (MGI Tech, Shenzen, China)
- MGI Sequencer DNBSEQ-T7™ (MGI Tech, Shenzen, China)
- MiniSeq® (Illumina Inc. California, USA)
- MiSeq® (Illumina Inc. California, USA)
- MiSeq® Dx (Illumina Inc. California, USA)
- MiSeq® FGx (Illumina Inc. California, USA)
- NextSeq® (Illumina Inc. California, USA)
- NovaSeq® 6000 (Illumina Inc. California, USA)
- other

If other, please define

18. Sequencing details #1 (please select one answer)*:
- Single-end
- Paired-end
- Not relevant

19. Sequencing details #2:
For the sequencing, the read length (bp) was set to be (expected read length)

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

21. Reads trimmed before upload (please select one answer)*:
[**Note**; this question refers to trimming performed actively by the participant (*i.e.* trimming performed automatically by your sequencing machine is not relevant for this question).
Ideally, no trimming should be performed.
As part of the analysis of the sequences subsequent to the deadline of the PT, trimming will be performed by application of the same tool for all submitted sequences.

Should trimming be an integrated part of your sequencing process (disregarding possible automatic trimming by your sequencing machine), please indicate with 'yes' to this question]

- Yes
- No

22. If the reads were trimmed, which tool was applied (in the following text field, please insert name and URL/link (if possible))

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

24. If applicable, how was the QC of the assemblies performe? Please mention the program (e.g., QUAST) and the QC metrics (e.g. N50, L50 etc.) used.

ANALYSIS of sequences

25. For determining the MLST of the sequenced DNA, how was the analysis performed (please select one answer)?*

    - MLST-analysis was performed on raw reads
    - MLST-analysis was performed on contigs
    - MLST-analysis was not performed

26. For determining the Plasmid replicon type, how was the analysis performed (please select one answer)?*

    - Plasmid replicon type analysis was performed on raw reads
    - Plasmid replicon type analysis was performed on contigs
    - Plasmid replicon type analysis was not performed

27. For determining antimicrobial resistance (AMR) genes present in the sequenced DNA, how was the analysis performed (please select one answer)?*

    - Analysis for AMR-genes was performed on raw reads
    - Analysis for AMR-genes was performed on contigs
    - Analysis for AMR-genes was not performed

28. For the detection of Multi Locus Sequence Type, 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, the version number and the URL. 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

29. For the detection of plasmid replicon type, 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, the version and the URL. If you used an in-house script, please specify the program
    c) Database: publicly available, commercial, or in-house database. If you used an available database, please report the name and version number. If you used an in-house database, please briefly describe the genes included.
    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.)

30. 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, the version and the URL. If you used an in-house script, please specify the program
    c) Database: publicly available, commercial, or in-house database. If you used an available database, please report the name and version number. If you used an in-house database, please briefly describe the genes included.
    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.)

31. 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, the version number and the URL. If you used an in-house script, please specify the program
    c) Database: publicly available, commercial, or an in-house database. If you used an available database, please report thename and version number. If you used an in-house database, please briefly describe the point mutations included.
    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.)

32. For the WGS-based prediction of antimicrobial resistance phenotypes, 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, the version number and the URL. If you used an in-house script, please specify the program
    c) Database: publicly available, commercial, or an in-house database. If you used an available database, please report the name and version number. If you used an in-house database, please briefly describe the sequences included
    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.)

SUBMITTED data files

33. Have the FASTQ-files (obtained non-assembled sequence data, file names as indicated in the protocol) been uploaded to the ScienceData-folder for bacterial cultures and DNA following the description in the PT-protocol? Please confirm by ticking off the response field* (yes/no)