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Version 1

2025 Oxford Nanopore Technologies (ONT) Sequencing Proficiency Testing Exercise V.1



Forked from [2024 GenomeTrakr Proficiency Testing exercise \(PulseNet Harmonized\)](#).

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We use this protocol and it's working

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FDA Foods Programme

Abstract

This SOP outlines guidelines on how to process the isolates for the 2025 ONT Proficiency Testing exercise.

- This SOP is applicable to all LFFM laboratory participating in the 2025 ONT Proficiency Testing exercise.

The FDA HFP/DFSG/GDAB will ship the following proficiency testing isolates in June 2025.

A	B	C
Strain ID	isolate_name_alias	Organism
GTVSS-001	CFSAN000189; 231742; PTC003; PTD003; CFSAN000189_B; CFSAN137036	<i>Salmonella enterica</i> serovar Bareilly
GTVSS-011	CFSAN023464; 869225- 3c; CFSAN137046	<i>Listeria monocytogenes</i>
GTVSS-014	CFSAN023469; 869226- 3c; CFSAN137049	<i>Listeria monocytogenes</i>
GTVSS-019	CFSAN044836; UNITO- 144; CFSAN137054	<i>Listeria innocua</i>
GTVSS-020	CFSAN051458; FDA858783-1-52; FNW19M81; FDA00010253; CFSAN137055	<i>Escherichia coli</i> O121
GTVSS-021	CFSAN068773; PR0330_EX_017; CFSAN137056	<i>Cronobacter sakazakii</i>
GTVSS-016	CFSAN030807	<i>Shigella sonnei</i>
GTVSS-025	CFSAN084952	<i>Pseudomonas fluorescens</i>

Completion of the entire proficiency test entails the following:

- LFFM participating laboratory generates sequencing data (fastq files) using the PT strains provided by FDA in accordance to FDA SOPs.
- Submission of sequencing records to the appropriate shared folder (box.com), see protocol.
- By participating in the 2025 ONT Proficiency Testing exercise, LFFM laboratories provide consent to use the PT exercise data in subsequent analysis and manuscript publications. Participants will be acknowledged for their contribution on any publication that might require processing data from the 2025 ONT PT exercise.

- Complete the participation survey at the end of the PT exercise.

Materials

1. Slant Cultures Materials and reagents

Trypticase Soy Agar (TSA) (or equivalent)

Trypticase Soy Broth (TSB) (or equivalent)

Brain Heart Infusion (BHI) Agar

Brain Heart Infusion (BHI) Broth

Disposable inoculating loops ( 1 µL or  5 µL)

 Ethanol 70% [Note: freshly prepared]

Permanent marker

Incubator range  25 °C to  37 °C

2. Recommended equipment for ONT sequencing.

	A	B	C
	Equipment	Product Number	Manufacturer
	Centrifuge (e.g., Eppendorf max speed 15,000rpm)	15881635	Fisher Scientific
	GridION sequencer		Oxford Nanopore Technologies (ONT)
	Plate centrifuge		Fisher Scientific
	Thermomixer (e.g., Eppendorf F2.0 Model)	15356551	Fisher Scientific
	PCR machine (e.g., T100 Thermal Cycler)	Any	Any
	Magnetic rack Invitrogen DynaMag -2 Magnet	10723874	Fisher Scientific
	Optional: Hula mixer	10548425	Fisher Scientific



	A	B	C
	Qubit fluorometer	16223001	Fisher Scientific
	Vortex (e.g., Fisherbrand mini vortexer)	Any	Any

3. Consumables for ONT sequencing.

	A	B	C
	Consumables	Product Number	Manufacturer
	Eppendorf DNA lo bind tubes 1.5ml (250)	EP0030108051-250EA	Sigma Aldrich
	Eppendorf DNA lo bind tubes 2ml (250)	EP0030108078	Sigma Aldrich
	96-well PCR plate semi-skirted (10)	AB0900	ThermoFisher Scientific
	Adhesive PCR Plate Seals (100)	AB0558	ThermoFisher Scientific
	Qubit Assay Tubes (500)	12037609	Fisher Scientific
	R10.4.1 Flowcell	FLO-MIN114	ONT

4. Reagents for ONT sequencing.

	A	B	C
	Reagents	Product Number	Manufacturer
	Nuclease-Free H2O	W4502-10×50ml	Sigma Aldrich
	Qubit 1x dsDNA HS Assay Kit (100)	Q33230	Fisher Scientific



A	B	C
Rapid Barcoding Kit 24 V14 or Rapid Barcoding Kit 96 V14	SQK-RBK114.24 or SQK-RBK114.96	Oxford Nanopore Technologies (ONT)
Ethanol, absolute (e.g., Fisher Bioreagents)	16606002	Fisher Scientific
Optional: Bovine Serum Albumin (BSA) (50 mg/ml)	AM2616	ThermoFisher Scientific
Optional: Wizard HMW DNA Extraction Kit	A2920	Promega
Optional: Monarch HMW DNA Extraction Kit	T3060S or T3050L	New England Biolabs
Optional: Nanobinds HMW	102-301-900	PacBio
Optional: Flow cell wash kit	EXP-WSH004	Oxford Nanopore Technologies (ONT)

Protocol materials

 Ethanol 70% [Note: freshly prepared]

Safety warnings

! **Biological Safety Warning:** *Escherichia/Shigella*, *Salmonella*, and *Listeria* strains are considered Level 2 biological agents by the U.S. Department of Health and Human Services. Use appropriate precautions when handling the vial or culture. Carry out laboratory work in a biological safety cabinet when applicable to ensure aseptic conditions and personal safety.



Before start

There are four sections in this protocol:

Section 1: Culture preparation of isolates.

Section 2: DNA Extraction

Section 3: Library Preparation and Sequencing

Section 4: Quality control and preliminary data analysis of Sequencing Data

Section 5: Data Transfer



Culture Preparation

2d 12h

1 *Salmonella*, *Shigella*, *Cronobacter sakazakii*, and *Escherichia* from slant cultures:

Day 1

Label the agar plate and broth tube (organism name, sample number, date, and initials), and document the isolate number for your records.

1.1 Wipe the outside of the slant tube with  Ethanol 70% [Note: freshly prepared] (be careful not to wipe the strain label).

1.2 Open the slant tube and gently touch a disposable  1-5 μL loop to a small amount of growth.

1.3 Streak the TSA agar plate for colony isolation (i.e., Quadrant or T-Streak).

1.4 Incubate the inverted TSA plate (agar side up , aerobically) at  37 °C for  Overnight or  18:00:00 .

18h

1.5 Day 2

Purity Check (Plate): colonies should appear similar in size, shape, and color.

1.6 After Purity check, pick an isolated colony and streak it onto a fresh TSA agar plate. Incubate (Aerobic) at  37 °C  Overnight or  24:00:00 .

1d

1.7 Day 3

Use the growth from this plate to make DNA templates of the PT strains.

2 *Listeria monocytogenes* and *L. innocua* slant cultures:

Day 1

Label the agar plate and broth tube (organism name, sample number, date, and initials), and document the isolate number for your records.



- 2.1 Wipe the outside of the slant tube with  Ethanol 70% [Note: freshly prepared] (be careful not to wipe the strain label).
- 2.2 Open the slant tube and gently touch a disposable  1-5 μL loop to a small amount of growth.
- 2.3 Streak the BHI agar plate for colony isolation (i.e., Quadrant or T-Streak).
- 2.4 Incubate the inverted BHI agar plate (agar side up , aerobically) at  37 °C for  Overnight or  18:00:00 .
- 2.5 **Day 2**

Purity Check (Plate): colonies should appear similar in size, shape, and color.
- 2.6 After Purity check, pick an isolated colony and streak it onto a fresh BHI agar plate. Incubate (Aerobic) at  37 °C  Overnight or  24:00:00 .

18h

2.7 Day 3

Use the growth from this BHI plate to make DNA templates of the *Listeria* PT strains.

3 *Pseudomonas fluorescens* from slant cultures:

Follow the same procedure than for *Salmonella*.

DNA Extraction

2m 5s

- 4 ****-** Each isolate for the proficiency testing exercise shall be processed as any routine isolate according to laboratory guidelines.
- 4.1 Perform DNA extraction according to lab's normal workflow described on GenomeTrakr and/or PulseNet SOPs.
- 4.2 **(1) Gram negative bacteria** (i.e. *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Serratia marcescens*, *Enterococcus faecalis*, *Bacillus subtilis*)
 - a. DNA is extracted using the Maxwell RSC cultured cells DNA kit with a Maxwell RSC instrument (Promega, Madison, WI) following the manufacturer's protocols for Gram-



negative bacteria with additional RNase treatment. **The protocol for the Maxwell RSC cultured cells DNA kit is in annex 1.**

b. DNA concentration is determined by Qubit 4 Fluorometer (Invitrogen, Carlsbad, CA) according to manufacturer's instructions.

4.3 **(1) Gram positive bacteria** (i.e. *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Clostridium botulinum*, etc)

a. pre-lysis treatment of the cells to rupture the wall (according to your own protocols for Gram positive bacteria)

b. Continue as described for Gram negative bacteria above.

4.4 Place the extracted DNA at 4-8 °C °C until needed. This step is required to avoid further shearing from freezing and thawing.

4.5 **DNA quantification** (Qubit HS Assay kit and Qubit fluorometer)

2m 5s

a. Using 1x dsDNA high sensitivity Qubit reagents, aliquot 198 µL per sample and 2 x 190 µL per standard.

b. Add 10 µL of each standard to 190 µL of Qubit reagent.

c. Add 2 µL of DNA extract to 198 µL of Qubit reagent.

d. Vortex for 00:00:05 then incubate at Room temperature for 00:02:00 2min.

e. Read on the Qubit.

f. Expected yield 50-120 ng/µl (optimum input concentration for library preparation is 200 ng total) - Yield will vary by organism > 11 ng/µl ng/µl is sufficient for 200 ng in 18 µL barcoding reactions.

Library Preparation and Sequencing

5 **** The sequencing run for the PT exercise shall be loaded as any routine run following established loading requirements.**

**** Control reference strains shall be included in the run when there is not enough isolates to fill a sequencing run.**

- 5.1 Perform library preparation according to lab's normal workflow described on FDA Human Foods Program ONT SOPs.

Protocol



NAME
Closing bacterial genomes using the Rapid Sequencing Kit (SQK-RBK114) from Oxford Nanopore Technologies (ONT)

CREATED BY
Narjol Gonzalez-Escalona

Preview

- 5.2 Sequencing with ONT does not require a sample sheet but you might be to do if desire as below:

A	B
barcode	alias
barcode13	sample01
barcode14	sample02
barcode15	sample03
barcode16	sample04
barcode17	sample05
barcode18	sample06
barcode19	sample07
barcode20	sample08

A	B
barcode21	sample09
barcode22	sample10
barcode23	sample11
barcode24	sample12

you can use this format to perform analysis using the epi2me lab software from ONT

5.3 Strain_ID or Sample_Name: Include in these fields the strain identifiers as it is indicated in Table 1, *do not modify these IDs*. You will also find this identifier in the tube of the slant culture.

A	B	C
Strain ID	isolate_name_alias	Organism
GTVSS-001	CFSAN000189; 231742; PTC003; PTD003; CFSAN000189_B; CFSAN137036	Salmonella enterica serovar Bareilly
GTVSS-011	CFSAN023464; 869225-3c; CFSAN137046	Listeria monocytogenes
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GTVSS-020	CFSAN051458; FDA858783-1-52; FNW19M81; FDA00010253; CFSAN137055	Escherichia coli
GTVSS-021	CFSAN068773; PR0330_EX_017; CFSAN137056	Cronobacter sakazakii
GTVSS-016	CFSAN030807	Shigella sonnei
GTVSS-025	CFSAN084952	Pseudomonas fluorescens



Quality of Sequencing Data

- 6 Perform quality control and preliminary data analysis of Sequencing Data according to lab's normal workflow described below:

Protocol

NAME

Quality control and preliminary data analysis of Sequencing Data: GalaxyTrakr ONT workflow

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Narjol Gonzalez-Escalona

Preview

Data Transfer

7 Data transfer

After checking the quality of your records, transfer the data with **acceptable quality** according to GenomeTrakr guidelines via the HHS Box platform.

7.1 Sharing a sequence data via the HHS Box platform

A folder will be generated within the HHS Box platform to which the uploader(s) will be invited to the platform where they can then upload the fastqs sequence data.

Below is an example of the Box interface through which participants will upload sequence data. Participants can either drag and drop the required files or folder with the files or do so via the Upload button.

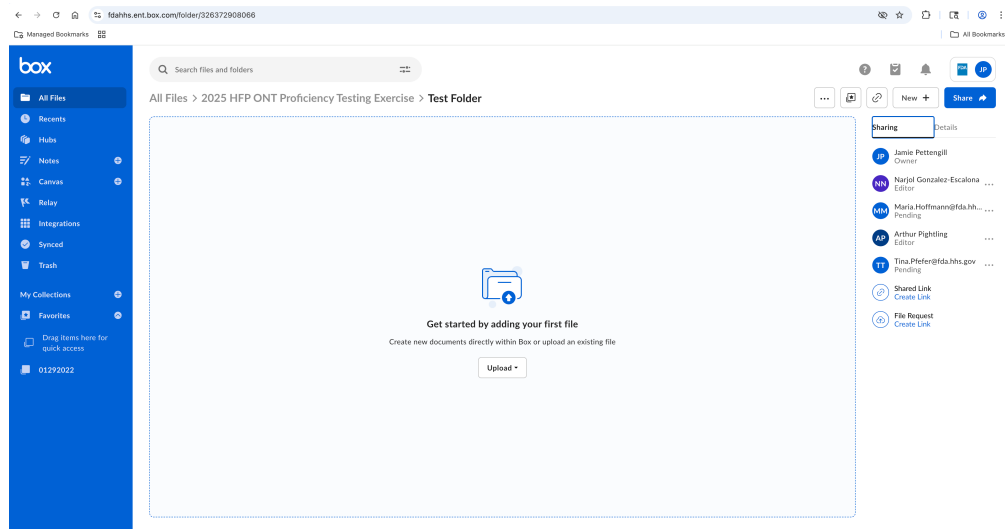


Figure 1. Example of the Box interface through which participants will upload files.