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2025 Oxford Nanopore Technologies (ONT) Sequencing Proficiency Testing Exercise V.2

Version 1 is 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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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	*Salmonella enterica serovar Bareilly*
	GTVSS-011	CFSAN023464; 869225-3c; CFSAN137046	*Listeria monocytogenes*
	GTVSS-014	CFSAN023469; 869226-3c; CFSAN137049	*Listeria monocytogenes*
	GTVSS-019	CFSAN044836; UNITO-144; CFSAN137054	*Listeria innocua*
	GTVSS-020	CFSAN051458; FDA858783-1-52; FNW19M81; FDA00010253; CFSAN137055	*Escherichia coli O121*
	GTVSS-021	CFSAN068773; PR0330_EX_017; CFSAN137056	*Cronobacter sakazakii*
	GTVSS-016	CFSAN030807	*Shigella sonnei*
	GTVSS-025	CFSAN084952	*Pseudomonas fluorescens*

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 . 18h

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 .

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 [M] 50-120 Mass Percent (optimum input concentration for library preparation is 200 ng total) - *Yield will vary by organism >* [M] 11 Mass Percent *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.*

*** **The minKNOW software version should be v25.05.14 or later.***

5.1 Perform library preparation according to lab's normal workflow described on FDA Human Foods Program ONT SOPs. **Since there are only 8 isolates for this PT exercise, we recommend running them in duplicate on the same flow cell.**

Protocol

NAME

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

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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

	A	B
	barcode20	sample08
	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
	GTVSS-014	CFSAN023469; 869226-3c; CFSAN137049	Listeria monocytogenes
	GTVSS-019	CFSAN044836; UNITO-144; CFSAN137054	Listeria innocua
	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

	A	B	C
	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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Preview

Data Transfer

7 **Data transfer**

After checking the quality of your records, transfer the data with **acceptable quality** (passing run and assembly metrics) according to GenomeTrakr guidelines via the HHS Box platform.

7.1 **Sharing a sequence data via the HHS Box platform:**

Please send an email to our FDA team at **tina.pfefer@fda.hhs.gov** and let us know that you have finished your ONT PT Exercise and are ready to submit the data.

Upon receipt of your email, the FDA team will create a folder for you within the HHS Box platform.

You will then receive a link inviting you to access your folder on this platform, where you will upload the **fastq** sequence data, **Excel sheet** with your ***in silico*** **results**, and the **HTML report** produced at the end of the run.

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.

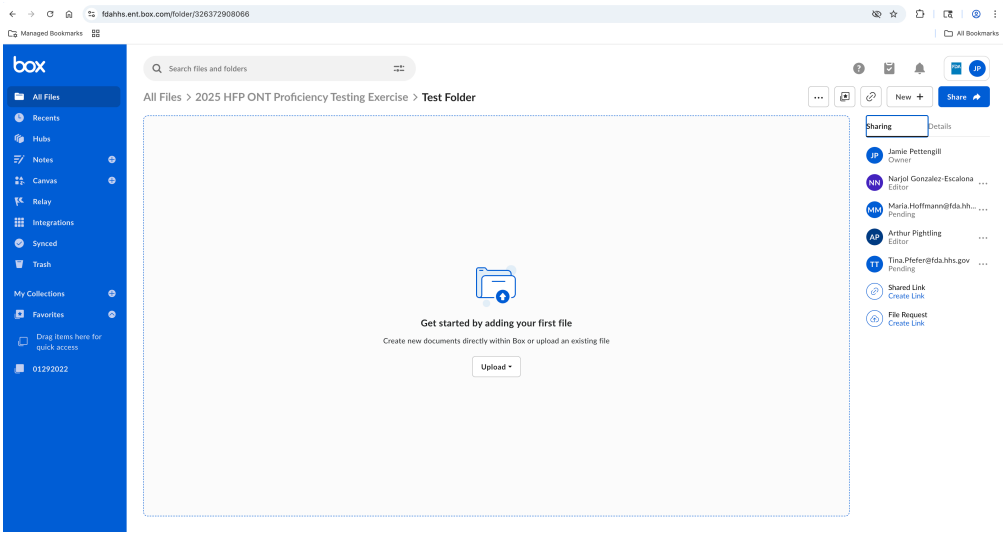

Figure 1. Example of the Box interface through which participants will upload files.