

STANDARD OPERATING PROCEDURE FOR THE PULSENET WGS PROFICIENCY TESTING PROGRAM			
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1. **PURPOSE:** To describe the procedure for the performance of WGS proficiency testing within PulseNet USA network. This is part of the PulseNet WGS QA/QC program.
2. **SCOPE:** This procedure applies to PulseNet USA certified personnel at participating laboratories who perform proficiency testing. Successful completion of the PulseNet proficiency test for WGS also satisfies the GenomeTrakr proficiency testing requirement.
3. **DEFINITIONS/TERMS:**
 - 3.1 **7-gene MLST:** Multi-Locus Sequence Typing scheme based on seven conserved housekeeping genes.
 - 3.2 **Allele:** One of two or more alternative forms of a gene that arise by mutation and are found at the same place on a chromosome.
 - 3.3 **ANI:** Average Nucleotide Intity. A computational method that compares an unknown query sequence to a reference sequence to determine the genetic similarity of shared sequences and proportion of bases aligned.
 - 3.4 **AR:** Antimicrobial Resistance
 - 3.5 **Analysis-certified:** An individual or laboratory that is certified for checking WGS data quality, performing analysis and publishing to the PulseNet National Database and NCBI using PN 2.0.
 - 3.6 **Assembly Length:** The length of the assembled genome, that is calculated by adding up the lengths of the individual contigs forming the assembly. In the PN 2.0 platform the assembly length is expressed in base pairs.
 - 3.7 **BaseSpace:** Illumina cloud-based computing environment for next generation sequencing data analysis and management, including data sharing.
 - 3.8 **CDC:** Centers for Disease Control and Prevention
 - 3.9 **cgMLST:** Core Genome Multi-Locus Sequencing Typing
 - 3.10 **Core genome:** Genes shared by a vast majority of strains of the same species (e.g., >99% of *Listeria monocytogenes* strains).
 - 3.11 **Coverage:** The average number of reads that include a given nucleotide in the reconstructed sequence.
 - 3.12 **Critical Quality Metrics:** Genus & species (ANI percent aligned, ANI score), average de novo coverage, average quality (Q score), assembly length, MIDAS secondary coverage, percent core alleles called, and allele difference compared to reference (wet lab PT only). Failing to meet the minimum thresholds/acceptable range for any one of these metrics will result in a failure of PT.
 - 3.13 **CSV:** Comma Separated Value
 - 3.14 **DCIPHER:** Data Collation and Integration for Public Health Events
 - 3.15 **De Novo Assembly:** A sequence assembly generated from the short raw reads without the use of a reference genome.
 - 3.16 **Dry Lab Proficiency Test:** The annual challenge testing the participant's skills in analyzing sequence data using PN 2.0.
 - 3.17 **Fastq (wet lab)-certified:** An individual or a laboratory that is certified in laboratory methods for WGS.
 - 3.18 **Fastq file:** A text-based file format for storing both sequence and its corresponding quality scores.
 - 3.19 **FDA:** Food and Drug Administration
 - 3.20 **FTP:** File Transfer Protocol; a standard network protocol used to transfer computer files from one host to another host via Internet.
 - 3.21 **GZ:** Gnu Zipped. A file format that is based on GNU ZIP compression algorithm, also known as GZIP compression technology.

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- 3.22 **“In-house fastq files”**: sequencing results generated by the participating laboratory. This is a part of the proficiency testing program where laboratories produce fastq.gz files containing sequencing data.
- 3.23 **LRM**: Local Run Manager
- 3.24 **MB**: Mega Base (1 million base pairs)
- 3.25 **MIDAS**: Metagenomic Intra-species Diversity Analysis System. Integrated computational pipeline for quantifying bacterial species abundance and coverage from shotgun metagenomes based on blast alignment against a panel of universal single copy genes.
- 3.26 **NCBI**: National Center for Bio**te**chnology Information
- 3.27 **NextFlow**: Scientific workflow system that establishes standards for programmatically creating a series of dependent computational steps and facilitates their execution on various local and cloud-based resources.
- 3.28 **Organism-specific Database**: A PN 2.0 database, used for comparing isolates for surveillance. Part of the standard PulseNet workflow.
- 3.29 **Percent (%) Core Alleles Called**: Percentage of the genes detected from a given de novo assembly that belong to the core genome. 100% means that all core genes of the species were detected from the assembly.
- 3.30 **PN 2.0**: PulseNet 2.0. A customized cloud-based (cloud service provider Microsoft Azure) start-to-end automated sequence analysis workflow utilized by PulseNet USA. The workflow is composed of open-source tools that are integrated in a cohesive pipeline within a NextFlow framework.
- 3.31 **Proficiency Testing (PT)**: A mandatory annual assessment of the quality of the work being performed in PulseNet participating laboratories.
- 3.32 **Proficiency Test file evaluator (evaluator)**: A PulseNet Central staff member who evaluates and signs off on the proficiency test reports submitted by PulseNet participants
- 3.33 **Proficiency Testing Packet**: An electronic PDF file including a cover letter and the proficiency testing evaluation that is emailed to the primary PulseNet contact for the laboratory and the participant’s laboratory director.
- 3.34 **Proficiency Testing Report**: A report that contains the evaluation and results of the participant’s proficiency test. See example templates in Appendix PNQ09-1a and PNQ09-1b.
- 3.35 **PulseNet Area Laboratory**: Laboratory, designated by CDC, which has agreed to assume responsibility for additional PulseNet duties for laboratories within their support zone. The current Area Laboratories include CO, MI, MN, NY, TN, VA and WA.
- 3.36 **PulseNet Central**: PulseNet team at CDC comprised of the PulseNet Response and Outbreak Management Team (PulseNet@cdc.gov) and PulseNet NGS Activities (PulseNetNGSlab@cdc.gov).
- 3.37 **PulseNet/OutbreakNet SharePoint**: A closed, web-based collaboration application used for communication among PulseNet participants.
- 3.38 **PulseNet PT Submission Form**: An electronic form in DCIPHER that each participating laboratory is required to fill out and submit. This form captures the submitting laboratory’s general submission details, specifics pertaining to the PT run(s), QC, identification and genotyping results for the PT strains, results for the cluster detection challenge, and the library prep workbooks (or Run Details file for Clear Labs) generated for the PT sequencing run(s).
- 3.39 **QA/QC**: Quality Assurance/Quality Control
- 3.40 **Q score**: The sequence quality score for each individual base position in a sequence, indicating the accuracy of the base call. Phred scores are used, where $Q = -10\log(\text{Error Probability})$. The higher the quality score, the more reliable the base call. A Q30 means a 1 in 1000 likelihood of an incorrect base call at that position.
- 3.41 **Read**: A unit of continuous DNA sequence (based pairs) derived by sequencing a part of the fragmented target DNA.
- 3.42 **SOP**: Standard Operating Procedure

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- 3.43 **SRA:** Sequence Read Archive, database at NCBI which stores raw sequence data and alignment information.
- 3.44 **Wet Lab Proficiency Test:** The annual challenge testing the participant's skills in the laboratory procedure to generate fastq files.
- 3.45 **wgMLST:** Whole Genome Multi-Locus Sequence Typing
- 3.46 **WGS:** Whole Genome Sequencing

4. RESPONSIBILITIES

- 4.1 Individuals performing PulseNet-related work (i.e., sequencing and/or analyzing data in PN 2.0) must be certified before being able to participate in the proficiency testing program. Newly certified labs must be certified for at least two months before participating in the Proficiency Testing Program. See PNQ08 (SOP for WGS Certification of PulseNet Personnel) for information on becoming certified.
- 4.2 Certified individuals in PulseNet participating laboratories must participate in PulseNet proficiency testing annually as part of continuing in-house QA/QC. It is recommended that all certified individuals in a laboratory perform wet lab and dry lab proficiency testing in-house, but only one set of fastq files and one set of analyzed data per laboratory is selected and submitted to PulseNet Central for evaluation. ***GenomeTrakr participants must also share their BaseSpace projects with FDA.***
 - 4.2.1 **Fastq (lab)-certified individuals** will sequence 4 proficiency testing strains to generate in-house fastq files for submission and evaluation by PulseNet Central. Fastq files and Run Workbooks (Run Details file for Clear Labs) will be shared with PulseNet Central as outlined in the Procedure (Section 5) and the PT Instructions. **NOTE:** Laboratories not capable of culturing and sequencing *Campylobacter* are not required to process the *Campylobacter* strain if one is included in the annual PT panel. The remaining organisms are REQUIRED.
 - 4.2.2 **Analysis-certified individuals** will analyze the 4 fastq files generated for the proficiency test strains and the sequences designated for the cluster detection challenge using the PN 2.0 workflow. Results for the sequence QC, identification, genotyping, cluster detection, and entry of the designated metadata and general submission information will be recorded/uploaded on the PulseNet PT Submission Form in the required format outlined in the Procedure (Section 5) and the PT Instructions and submitted to PulseNet Central for evaluation.

5. PROCEDURE

- 5.1 Individuals at participating laboratories must perform the proficiency testing according to the current proficiency testing instructions that are emailed to primary PulseNet contacts and posted on SharePoint within the PulseNet Announcements forum.
- 5.2 Laboratories are required to include routine current and/or historical *Salmonella* and/or *E. coli* isolates on the same cartridge as the proficiency testing isolates in order to fill up the run. Acceptable library preparation kits, sequencing platforms, sequencing chemistries and DNA loading capacities are listed in Appendix PNQ09-2. **NOTE:** All 4 PT strains do not need to be on the same sequencing run.
- 5.3 At least one fastq-certified individual in a laboratory must perform **wet lab proficiency testing** using the complete set of 4 PT strains annually as part of in-house QA/QC. It is recommended that all fastq-certified individuals sequence at least one of the PT strains annually as part of in-house competency assessment. However, only one set of fastq files per laboratory should be chosen for submission through the PulseNet/GenomeTrakr BaseSpace projects or the PulseNetQA FTP site. **NOTE:** The

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fastq files **are required to have the .gz extension**. The PN 2.0 analysis pipeline only accepts gzip-compressed files, not plain .fastq files.

- 5.3.1 BaseSpace sharing of **the project containing fastq.gz files** with PulseNet Central as the collaborator using the email: PulseNet@cdc.gov.
 - 5.3.1.1 Initially stream the files to a separate QC project on BaseSpace that is not shared with CDC. **NOTE: Do not share the fastq.gz files until you know that the files pass critical quality metrics in the PN 2.0 analysis platform.**
 - 5.3.1.2 Files passing critical quality metrics can then be saved to a project shared with CDC. **NOTE:** Files should not be “copied” to a shared project (i.e., Dataset name should not have “copy” at the beginning).
 - 5.3.1.3 Use **a separate project for PT** named “<Lab ID>_Proficiency_Testing” where Lab ID is listed as the laboratory’s PulseNet ID (i.e. AK).
NOTE1: When setting up the project in BaseSpace, **use underscores** to separate the words; LRM does not allow the use of spaces. **NOTE2:** If you share your files on BaseSpace without using this project naming scheme, **points will be deducted.**
- 5.3.2 GenomeTrakr labs only: BaseSpace sharing of **the fastq.gz files** with FDA using the email: GnomeTrakr@fda.hhs.gov.
- 5.3.3 Laboratories not using BaseSpace: Separate instructions will be provided for accessing the PulseNetQA FTP site. Email PulseNet@cdc.gov to request the FTP access credentials. Create a separate folder on the ftp site named “<Lab ID>_Proficiency_Testing” where Lab ID is listed as the laboratory’s PulseNet ID (i.e. AK). **NOTE:** If you do not share files correctly on the FTP site, **points will be deducted.**
- 5.3.4 If you are unable to share the fastq.gz files with PulseNet Central using BaseSpace or the FTP site, please contact PulseNet@cdc.gov for alternative arrangements.
- 5.4 At least one analysis-certified individual in a laboratory must annually conduct **dry lab proficiency testing** by performing the PN 2.0 analysis of the fastq files generated for the 4 wet lab PT strains and the sequence set designated for the cluster detection challenge. It is recommended that all analysis-certified individuals in a laboratory perform the PN 2.0 analysis of the PT materials annually as part of in-house competency assessments. However, only one set of analysis results per laboratory should be chosen for submission.
 - 5.4.1 **The PulseNet PT Submission Form available in DCIPHER** is used to report general information about the PT sequencing runs, and results from the sequence QC, identification, genotyping, the cluster detection challenge, and metadata entry.
 - 5.4.1.1 Launch the PulseNet PT Submission Form either from the link provided in the PT announcement email or copy the link and paste into new browser screen.
 - 5.4.1.2 All questions marked with an asterisk are required.
 - 5.4.1.3 Each of the **4 PT strains** has a section for **recording critical QC metrics** from PN 2.0.
 - 5.4.1.3.1 Refer to Appendix PNQ09-3 for organism-specific minimum thresholds/acceptable ranges for critical QC metrics.
 - 5.4.1.3.2 In the event, the annual PT panel contains a *Campylobacter* strain, and the laboratory does not culture and sequence *Campylobacter*, select “No” for the question asking if your laboratory is capable of processing *Campylobacter* and respond “NA” for the questions associated with that strain.
 - 5.4.1.4 **Export the results for identification, genotyping and metadata entry for the 4 PT strains** performed in the organism-specific databases as Excel .csv files.

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Include all information fields and analyses that are listed in the PT instructions for each database.

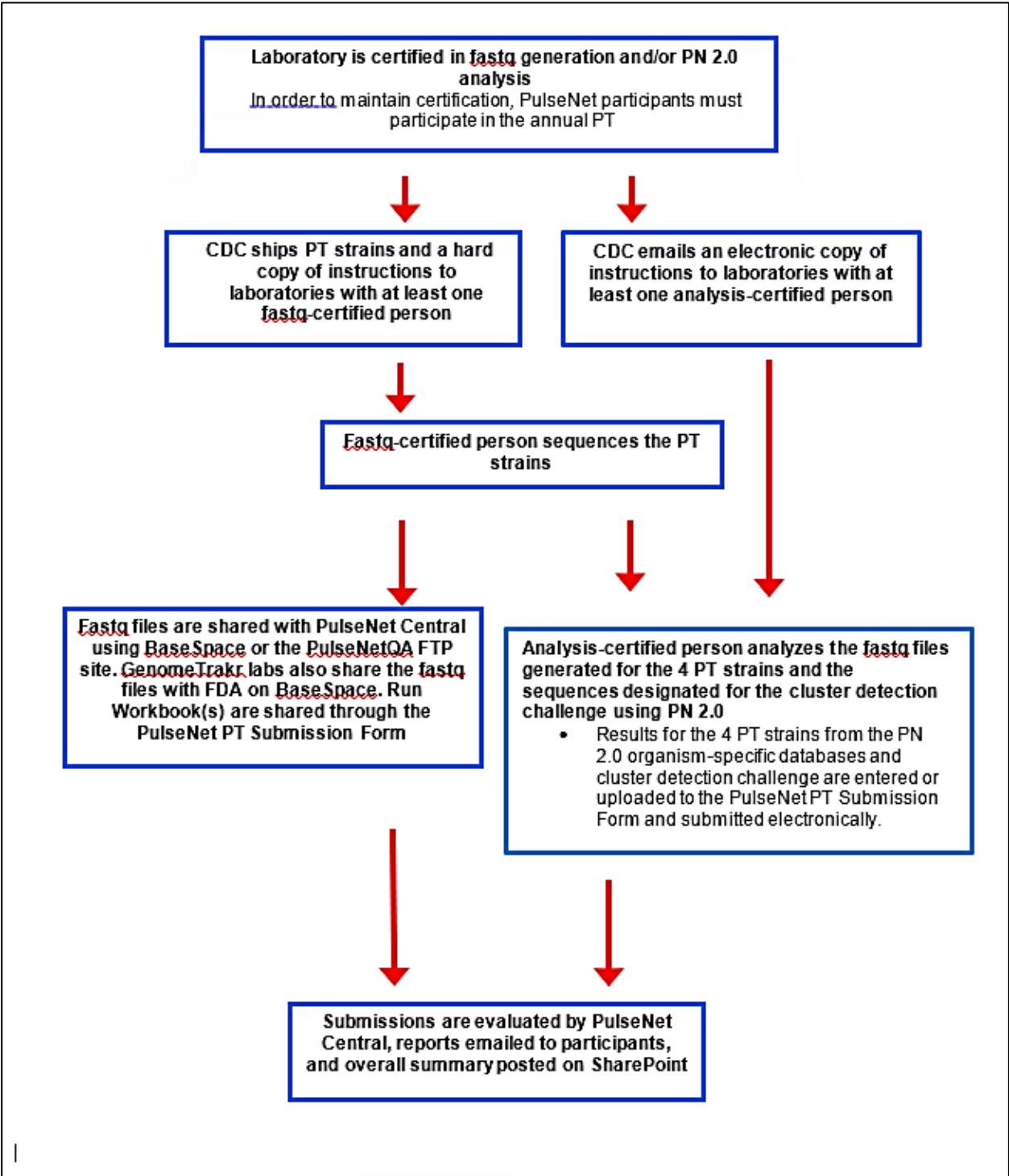
- 5.4.1.4.1 For each organism database, copy and paste the two Excel exports (metadata and resistance) into one organism-specific database tab (e.g., if both *E. coli* and *Shigella* are included in the panel, these results can be included in the same tab). **One Excel file** containing separate tabs for each organism database per laboratory should be submitted.
- 5.4.1.4.2 Name the file following the format: *LabID_PYear*, e.g., GA_PT25
- 5.4.1.4.3 The Excel file should be uploaded to the PulseNet PT Submission Form within the “Organism-specific Database Analysis Results and Metadata Entry” section.
- 5.4.1.5 The sequences designated for the **Cluster Detection Challenge** are accessible from NCBI/SRA and can be analyzed through PN 2.0 SRA pipeline. To record the results of the cluster detection challenge, fill out the “Cluster Detection” section of the PulseNet PT Submission Form.
 - 5.4.1.5.1 Refer to Appendix PNQ09-4 for guidelines performing local cluster detection.
- 5.4.1.6 After filling out all the required fields and attaching the required files (organism-specific database analyses, cluster detection dendrogram and sequencing Run Workbook(s) (Run Details file for Clear Labs) in the PulseNet PT Submission Form, submit the form by clicking “Submit”.
- 5.5 Send an email to PulseNet@cdc.gov to notify PulseNet Central that your fastq files are available for evaluation and you have submitted your PulseNet PT Submission Form. **NOTE:** Only send **one** email per laboratory.
 - 5.5.1 In the subject line of the email, include your PulseNet LabID and the words “WGS Proficiency Testing” (e.g., GA_WGS Proficiency Testing).
- 5.6 The fastq files and the dry lab analysis results submitted for proficiency testing must be generated by certified individuals. Results submitted by non-certified individuals will automatically fail the proficiency testing round.
- 5.7 All results must be submitted correctly by the submission deadline to avoid penalty. Laboratories that cannot meet the deadline may submit a request for extension to PulseNet@cdc.gov before the submission deadline.
- 5.8 Laboratories must pass proficiency testing annually to maintain PulseNet certification.
 - 5.8.1 **In order to pass the wet lab PT:**
 - 5.8.1.1 The fastq files for the 4 PT strains must meet the critical quality thresholds outlined in the Appendix PNQ09-3.
 - 5.8.1.2 A minimum of 85% of all points available must be accumulated.
 - 5.8.2 **In order to pass the dry lab PT:**
 - 5.8.2.1 The analyzed sequences for the 4 PT strains must meet the critical quality thresholds outlined in the Appendix PNQ09-3 for sequencing reads, assembly and allele calls and the required minimum metadata needs to be entered correctly. Points will be deducted for missing/incorrect assignment of serotype (if applicable), virulence profile (if applicable), AR profile, lineage (if applicable) and 7-gene MLST type. Points will be deducted if additional metadata fields are not filled out correctly.
 - 5.8.2.2 The sequences included in the cluster detection challenge must be correctly designated either belonging to a cluster (i.e., being highly related to adequate number of other sequences within the designated set to be considered a cluster) or being sporadic (entering “N/A does not cluster” if it is not highly related to another sequence in the designated set or the number of highly related sequences

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does not warrant designating a cluster). Cluster assignment must be based on the PulseNet guidelines for local cluster detection (PNQ09, Appendix 4). Missing 4 or more assignments will result in a failing score.

- 5.8.2.3 A minimum 85% of all points available must be accumulated.
- 5.8.3 The evaluator will email the PT packet consisting of the cover letter (Appendix PNQ09-5) and the PT reports (Appendices PNQ09-1a and PNQ09-1b) to the participant(s), the main PulseNet contact, the laboratory director and other necessary personnel (e.g., a QA/QC manager) designated by the submitting lab within the PulseNet PT Submission Form. If the participating laboratory is also a GenomeTrakr lab, the PT reports are also emailed to the GenomeTrakr PT contact(s).
- 5.8.4 Laboratories that fail the wet lab PT or the PN 2.0 analysis of the 4 generated PT sequences must resubmit the proficiency testing results for that pathogen again using the same strain(s). A new set of sequences for the cluster analysis challenge will be provided to those laboratories that fail the cluster detection module. The evaluator will contact the submitter directly via email to ask for a resubmission. Resubmission due dates will be determined by the evaluator.
- 5.8.5 Laboratories that fail a round of PT three times in a row will lose their certification. The evaluator will email a failure notification letter (Appendix PNQ09-6) to the participant, the primary PulseNet contact, the laboratory director, the appropriate PulseNet Area Lab, and the GenomeTrakr PT contact, if the participating laboratory is also a GenomeTrakr laboratory. Individuals at those laboratories must submit new certification files and pass certification again before reinstatement of their certification. Please see PNQ08 for PulseNet WGS Certification information.
- 5.8.6 CDC has the right to revoke certifications at any time.

6. FLOW CHARTS:



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7. RELATED DOCUMENTS:

Document Number	Title
PNL33	DNA Extraction and QC SOP
PNL34	Illumina Nextera XT Library Preparation SOP
PNL35	Illumina DNA Prep Library Preparation SOP
PNL36	Qiagen QIAseq FX Library Preparation SOP
PNL37	KAPA HyperPlus Library Preparation SOP
PNL38	Sequencing on the MiSeq SOP
PNL39	Sequencing on the MiniSeq SOP
PNL40	Sequencing on the iSeq SOP
PNL42	NEBNext Ultra II FS Library Preparation SOP
PNL43	Illumina DNA PCR-Free Library Preparation SOP
PNL44	Illumina DNA Prep Rapid Quarter Volume Library Preparation SOP
PND05.JA1	Illumina Sequence Data QC SOP
PND05	PN 2.0 Surveillance Database Workflow SOP
Provided via the email announcement	PulseNet WGS PT Instructions/ Supplementary Instructions
SharePoint: Library of PulseNet Documents/PulseNet 2.0 Reference Documents	PN2.0 Dictionary
SharePoint: Library of PulseNet Documents/QuickTips	2025-05-16_ClusterDetection

8. REFERENCES:

- 8.1 Timme, R.E., Lafon, P.C., Balkey, M., Adams, J.K., Wagner, D., Carleton, H., Strain, E., Hoffmann, M., Sabol, A., Rand, H., Lindsey, R., Sheehan, D., Baugher, J.D., Trees, E. (2020) Gen-FS coordinated proficiency test data for genomic foodborne pathogen surveillance, 2017 and 2018 exercises. Sci Data 7:402. doi: 10.1038/s41597-020-00740-7.

9. CONTACTS:

- 9.1 CDC PulseNet Response and Outbreak Management Team:
(404) 639-4558
PulseNet@cdc.gov
- 9.2 CDC PulseNet NGS Activities:
(404) 718-4185
PulseNetNGSlab@cdc.gov
- 9.3 GenomeTrakr:
Maria Balkey
(240) 402-4946
Maria.Balkey@fda.hhs.gov

10. AMENDMENTS:

- 10.1 2017-02-15 changes:
- Updated Quality Metrics thresholds for Average Read Quality (≥ 30), Assembly-based calls: Multiple Alleles (now shown in red), and Percent Core Present (now shown in red) in Appendix 9-4 Table.
- 10.2 2017-12-15 changes:

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- Updated SOP to include *Vibrio spp*
- Minor grammatical edits
- Updated Definitions for Proficiency Test evaluators, BaseSpace, WGS PT Reports, Category A and Category B infectious substances
- Updated Shipping requirements
- Updated duties outlined in Sections 5.2.3.4-5.2.3.6
- 10.3 2018-2-05 changes:
 - Updated Definitions to include Category A, Category B infections substances, Proficiency Test Report, and wgMLST.
 - Updated Proficiency Test Report Template in Appendix PNQ09-2.
 - Added language to include testing of *Vibrio spp*.
 - Revised Table in PNQ09-5 to include metrics for *Vibrio spp*.
 - Removed section 4.5.2 regarding duties of database and laboratory reviewer, revised to include only one reviewer.
 - Removed requirement to include labels identifying contents (i.e. 1x1.0 ml *Escherichia coli* O157:H7, 1 x 1.0 ml *Salmonella enterica* serotype Typhimurium). Replaced with requirement for Packing slips.
 - Removed requirement to sequentially number participant addresses or packages for Scireson. Only the SET booking numbers are required.
 - Added details to file submission in section 5.3.3.
 - Updated file evaluation process in section 5.4.3.
 - Updated Shipping contact information
- 10.4 2019-3-27 changes:
 - Updated the SOP reference in section 5.2.2 from PNL32 to PNL38.
 - Updated the Quality Metrics table in PNQ09-5
 - Added CDC Technology Transfer Office to Contacts
- 10.5 2020-3-16 changes:
 - Removed the CDC in-house procedure for planning and preparing PTs and evaluating submissions from PNQ09. This procedure is now outlined in an in-house SOP.
 - Added analysis PT
 - Added PulseNet Proficiency Testing Submission Template (Appendix PNQ09-2)
 - Change in policy: a lab will now lose its certification status for a particular organism after failing three attempts of Proficiency Testing
 - Added Appendix PNQ09-03 listing acceptable library prep kits and DNA loading capacities
 - Updated the critical quality metrics in the Appendix PNQ09-4 to be aligned with the RefID database workflow. HqSNP difference compared to CDC sequenced reference was removed as a critical quality metric for the wet lab PT. Minimum average Q score requirement was increased from 28.0 to 30.0.
- 10.6 2021-2-23 changes:
 - Added the cluster detection challenge to the Analysis PT
- 10.7 2022-3-25 changes:
 - Clarified the data sharing instructions
 - Clarified the bundle file naming instructions
 - Added the related Documents section #7
 - Updated the reports and the submission template in Appendices PNQ09-1 and PNQ09-2, respectively

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- Updated the Appendix PNQ09-3 Acceptable Library Preparation Kits, Sequencing Platforms, Sequencing Chemistries and Validated DNA Loading Capacities

10.8. 2023-3-24 changes

- Proficiency Testing Submission Template in Excel was replaced by E-PT Survey Template utilizing Google Forms
- Overall pass/fail scoring of the wet lab and dry lab PTs was changed from organism-specific to a single wet lab (fastq) and a single dry lab (analysis) PT. A minimum of 85% of the total of 200 points available is required for passing each of the wet lab and the dry lab PT.

10.9. 2024-03-06 changes

- E-PT Survey Template utilizing Google Forms was replaced by the PulseNet PT Submission Form in DCIPHER.
- Added PNL44 Rapid Library Prep SOP to related documents section
- Added requirement for naming PT projects on BaseSpace in the format “*Lab ID*_proficiency testing”.

10.9. 2025-07-25 changes:

- Replaced all references to the BioNumerics workflow with the PN 2.0 workflow. Results submission using BioNumerics bundle files was replaced with CSV file exports from PN 2.0. Sequences belonging to the cluster detection challenge are not provided as a bundle file anymore; instead, the participants access the relevant sequences from NCBI/SRA for analysis.
- Specified that the submitted fastq files are required to have the .gz extension.
- The PulseNet PT Submission Form in DCIPHER was updated to reflect the PN 2.0 workflow.
- Removed the requirement to share runs. Only sharing the sequences in a BaseSpace project/ftp site is required.
- Added the updated SOP PND05 (PN 2.0 workflow) and the new job aid PND05.JA1 (Illumina data QC, former SOP PNQ07) in the related documents list.
- Added MiSeq i100 to the Appendix PNQ09-2 as an acceptable sequencing platform.
- Updated the Appendix PNQ09-3 with thresholds applicable to PN 2.0.
- Added the Appendix PNQ09-4: Guidelines for local cluster detection. Former appendixes PNQ09-4 and 5 are now PNQ09-5 and 6.
- Updated Appendixes PNQ09-5 (Cover letter) and PNQ09-6 (Repeated failure and loss of certification) to reflect the fact that certifications are not organism-specific anymore. Instead, one fastq certification panel and one analysis certification covers all PulseNet organisms.

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11. APPROVAL SIGNATURES:

Approved By: _____ Date: _____
QA/QC Personnel

Approved By: _____ Date: _____
PulseNet Response and Outbreak Management Team Lead

Approved By: _____ Date: _____
PulseNet NGS Activities Technical Lead

Approved By: _____ Date: _____
PulseNet Reference Outbreak Surveillance Team Lead

Appendix PNQ09-1a. WGS Proficiency Testing Example Report Template for the Wet Lab PT (Fastq file Generation)

PulseNet Proficiency Testing Report for WGS Wet Lab (FASTQ Generation) - 2025			
Organism: <i>Escherichia/Shigella</i>			
PT Strain: <i>Shigella dysenteriae</i> ESP25-3026		Date strains shipped: 9/10/2025	Date results received:
Laboratory:			
I. Critical Quality Metrics (Pass/Fail 40 pts)	Possible Points	No. of Points Received	Comments
A. Average quality (≥30.0)	Pass or Fail	Pass	
B. MIDAS secondary coverage (≥10)	Pass or Fail		
C. Average denovo coverage (≥40x)	Pass or Fail		
D. Genus / species (<i>Escherichia coli</i>)	Pass or Fail		
E. Length (MB) - strain-specific ±5% of Reference Sequence (4.63 MB (4.46-4.82))	Pass or Fail		
F. Alleles called core present (≥95%)	Pass or Fail		
G. Number of cgMLST allele differences compared to reference (≤3)	Pass or Fail		
Pass/Fail Points	40	40	
Note: Items in Section I are in Pass/Fail format. Failure of any of these metrics will result in a request to repeat submission for that organism.			
II. Additional Sequencing Criteria (5 pts)			
A. Average read length			
600 cycle chemistry: ≥270 bp	3	3	
500 cycle chemistry: ≥225 bp			
300 cycle chemistry: ≥135 bp	2	2	
B. Fastq files named correctly	5	5	
Total Additional Sequencing Criteria Points	5	5	
Total <i>Escherichia/Shigella</i> Points	45	45	
WGS Performed by: Results Submitted by: Library prep kit used: Sequencing platform used: Sequencing chemistry used: Sequencing run genome load: Comments: Nice work!			
< > Escherichia Salmonella Vibrio Listeria FINAL RESULTS +			

2025 PulseNet Proficiency Testing Report for WGS Wet Lab			
Summary report			
III. Total Score Received Per Organism (180 pts)	Possible Points	No. of Points Received	Comments
A. <i>Escherichia/Shigella</i> Score	45	45	
B. <i>Salmonella</i> Score	45	45	
C. <i>Vibrio</i> Score	45	45	
D. <i>Listeria</i> Score	45	45	
IV. Additional Points Received for Correct Generation and Submission of Results (20 pts)			
A. PT isolates sequenced on a full cartridge (medium/large volume platforms/cartridges (Micro, v2 300c, v2 500c, v3, MiniSeq, NextSeq); 2 pts deducted for every missing 5 MB; Small volume cartridges/platforms (Nano, iSeq, Clariflex); 5 pts deducted for every missing 3 MB)	10	10	
B. Results correctly submitted to BaseSpace/FTP/Other sharing method	5	5	
C. Submission email sent	5	5	
V. Total Points	200	200	
VI. Total Percentage		100.0%	
Overall Proficiency Testing Result	Pass or Fail	Pass	
Overall Comments: Nice work!			
All metrics in Section I in each organism-specific component of the PT are in Pass/Fail format. If you fail any one of these sections, you fail the proficiency testing round. Critical quality metrics/acceptable ranges for PT are listed in the PulseNet proficiency testing SGP appendix PNQ09-3. A passing score is ≥85% (≥170/200).			
Evaluation Performed By: <u>Eija Trees, etj3@cdc.gov</u> Date:			
Approved By: <u>Ashley Sabol, asu3@cdc.gov</u> (404) 633-2947 Date:			
< > Escherichia Salmonella Vibrio Listeria FINAL RESULTS			

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Appendix PNQ09-1b. WGS Proficiency Testing Example Report Template for the Dry Lab PT (PN 2.0 Analysis)

	A	B	C	D	E	F
PulseNet Proficiency Testing Report for WGS Dry Lab (PN 2-0 Analysis) - 2025						
<i>Escherichia/Shigella</i>						
Organism database:	<i>Escherichia Shigella</i>					
PT Strain:	<i>Shigella dysenteriae</i> ESP25-3026					
Laboratory:	Date strains shipped:	3/10/2025	Data results received:			
Analysis Critical Pass/Fail Metrics (28 pts)	Possible Points	No. of Points Received	Comments			
A. Genus/species: <i>Escherichia coli</i>	Pass or Fail	Pass				
B. Average quality: ≥30.0	Pass or Fail					
C. MIDAS secondary coverage: ≤1.0	Pass or Fail					
D. Average de novo coverage: ≥40x	Pass or Fail					
E. Length (MB) – Strain-specific ±5% of Reference Sequence: 4.63 MB (4.46–4.32)	Pass or Fail					
F. Alleles called core present: ≥85%	Pass or Fail					
G. Resistance – Serotype and Phage: <i>serotype=untyped</i>	Pass or Fail					
Pass / Fail Points	28	28				
Note: Items in Section I are in Pass/Fail format. Failure of any of these metrics will result in a request to repeat submission for that organism.						
I. Additional Analysis Criteria (10 pts)						
A. Genotyping (5 pts)						
A1. Serotype_wgs: O12:	1	1				
A2. Virulence Profile/Pathotype – Consistent with Reference Sequences: eae, ehc, EPEC/Shigella	1	1				
A3. AR Profile – Consistent with Reference Sequence: uhpT_E359D, drfA14, glpT_E448K, perB_Y358N,qsrS1_blsTEM-1, splH(β-lid), spl(S ⁺)-lb, mlr2 (fosfomicin, trimastoprim, colistin, quinolone, beta lactam, streptomycin, rifampicinamide)	1	1				
A4. T-gene MLST – Consistent with Reference Sequence; No ST assigned (check allele not defining, most similar to allele 3)	1	1				
A5. Toxin_wgs: stx-	1	1				
B. Metadata Fields (4 pts) Provided metadata should be entered correctly in the organism-specific database						
B1. Source site entered correctly	1	1				
B2. Patient age entered correctly	1	1				
B3. Patient sex entered correctly	1	1				
B4. Collection date entered correctly	1	1				
C. Metadata/Resistance Export (1 pt)						
C1. Correct information fields included in the export file	1	1				
Total Additional Analysis Criteria Points	10	10				
Total Escherichia/Shigella Analysis Score	38	38				
Analysis Performed by:						
Results Submitted by:						
<	>	Escherichia	Salmonella	Vibrio	Listeria	Cluster Detection FINAL RESULTS

2025 PulseNet Proficiency Testing Report for WGS Dry Lab (PN 2.0 Analysis)			
Summary Report			
IV. Total Score Received Per Organism (152 pts)	Possible Points	No. of Points Received	Comments
A. <i>Escherichia</i> Analysis Score	38	38	
B. <i>Salmonella</i> Analysis Score	38	38	
C. <i>Vibrio</i> Analysis Score	38	38	
D. <i>Listeria</i> Analysis Score	38	38	
V. Total Cluster Detection Score (38 pts)	38	38	
VI. Submission of Results (10 pts)			
A. Results submitted by the deadline	5	5	
B. Submission email sent	5	5	
VII. Total points	200	200	
VIII. Total Percentage		100.0%	
Overall Proficiency Testing Result	Pass or Fail	Pass	
Comments: Nice work!			
Critical quality metrics/acceptable ranges for PT are listed in the PulseNet Proficiency Testing SOP appendix: PNQ03-3. PulseNet guidelines for local cluster detection are outlined in the Appendix: PNQ03-4. A passing score is ≥85% (≥170/200) with no failures in the metrics with automatic Pass/Fail criteria. Proficiency Testing Evaluation and Approval: Evaluation Performed By: _____ Date: _____ Eija Trees, et3@cdc.gov Approved By: _____ Date: _____ Kelley Hise, khb5@cdc.gov (404) 639-0704			

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Appendix PNQ09-2. Acceptable Library Preparation Kits, Sequencing Platforms, Sequencing Chemistries and Validated DNA Loading Capacities

Nextera XT¹, Illumina (PNL34)

Illumina Sequencing Kit	DNA Load (Mb)
MiSeq v2, 300	60
MiSeq v2, 500	80
MiSeq v3, 600 ² no <i>E. coli/Shigella/Vibrio</i>	200
MiSeq v3, 600 ² <i>E. coli/Shigella/Vibrio</i>	175
MiSeq Micro, 300	35
MiSeq Nano, 500	13
MiniSeq Mid Output	60
MiniSeq High Output	100

¹300 cycle chemistry not acceptable for *Escherichia*, *Shigella* or *Vibrio*

²DNA loads for v3 600 cycle cartridges are based on sequencing runs of 500 cycles using this chemistry

Illumina DNA Prep (DNA Flex) (PNL35)

Illumina Sequencing Kit	DNA Load (Mb)
MiSeq v2 300	90
MiSeq v2 500	100
MiSeq v3 600 ¹ no <i>E. coli/Shigella/Vibrio</i>	200
MiSeq v3 600 ¹ <i>E. coli/Shigella/Vibrio</i>	175
Micro 300 no <i>E. coli/Shigella/Vibrio</i>	35
Micro 300 <i>E. coli/Shigella/Vibrio</i>	30
Nano 500	13
Nano 300 no <i>E. coli/Shigella/Vibrio</i>	13
Nano 300 <i>E. coli/Shigella/Vibrio</i>	10
MiniSeq Mid Output	60
MiniSeq High Output	100
iSeq <i>E. coli/Shigella/Vibrio</i>	25
iSeq no <i>E. coli/Shigella/Vibrio</i>	35
NextSeq 1000/2000 P1 300	400
ClearLabs Clear Dx (iSeq)	22
MiSeq i100 25M 300	100 ²

¹DNA loads for v3 600 cycle cartridges are based on sequencing runs of 500 cycles using this chemistry.

²Not validated yet. Estimation based on the loading capacity on the legacy MiSeq but the actual loading capacity likely to be higher.

QIAseq FX, Qiagen (PNL36)

Illumina Sequencing Kit	DNA Load (Mb)
MiSeq v2 300	80
MiSeq v2 500	100

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KAPA HyperPlus, Roche (PNL37)

Illumina Sequencing Kit	DNA Load (Mb)
MiSeq v2 300, no <i>E. coli/Shigella/Vibrio</i>	80
MiSeq v2 300, <i>E. coli/Shigella/Vibrio</i>	75
MiSeq v2 500	100

NEBNext Ultra II FS (PNL42)

Illumina Sequencing Kit	DNA Load (Mb)
MiSeq v2 300, no <i>E. coli/Shigella/Vibrio</i>	90
MiSeq v2 300, <i>E. coli/Shigella/Vibrio</i>	75
MiSeq v2 500	100

Illumina DNA PCR-Free (PNL43)

Illumina Sequencing Kit	DNA Load (Mb)
MiSeq v2 300	90
MiSeq v2 500	100

PulseNet Rapid SOP (PNL44)

Illumina Sequencing Kit	DNA Load (Mb)
MiSeq v2 300	80
MiSeq v2 500	100
MiSeq i100 300	100 ¹

¹Not validated yet. Estimation based on the loading capacity on the legacy MiSeq but the actual loading capacity likely to be much higher.

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Appendix PNQ09-3. Critical Quality Metric Thresholds/Acceptable Ranges for PulseNet Proficiency Testing

QC metric terminology as listed in the Isolate Sample form > Processes and Metrics > Summary. In the parenthesis is listed the terminology as found under the respective Process in the Isolate Sample form > Processes and Metrics > Processes.

Organism	AvgDeNovoCoverage (cleanedAverageDepth) ¹	AverageQuality_Qscore (TrimmedCombinedAvgQC)	Length (cleanedLength) (bp)	ANI Percent Aligned	ANI_Score	MIDASSecondaryCoverage (secondaryCoverage_coverage)	AllelesCalledCorePercent (corePercentage)	cgMLST Allele Difference to the Reference	AvgReadLength (trimmedCombinedMeanReadLength) (bp)
<i>Listeria monocytogenes</i>	≥ 20x	≥ 30	2800000-3199999	≥ 75	≥ 92	≤ 1	≥ 95	≤ 3	≥ 135 (300c) ≥ 225 (500c) ≥ 270 (600c)
<i>Escherichia coli</i> and <i>Shigella</i> spp.	≥ 40x	≥ 30	4200000-5999999	≥ 70	≥ 95	≤ 1	≥ 85	≤ 3	≥ 135 (300c) ≥ 225 (500c) ≥ 270 (600c)
<i>Salmonella</i> spp.	≥ 30x	≥ 30	4300000-6200000	≥ 70	≥ 92	≤ 1	≥ 85	≤ 3	≥ 135 (300c) ≥ 225 (500c) ≥ 270 (600c)
<i>Campylobacter</i> spp.	≥ 20x	≥ 30	1400000-2200000	≥ 70	≥ 92	≤ 1	≥ 85 ²	≤ 3	≥ 135 (300c) ≥ 225 (500c) ≥ 270 (600c)
<i>Vibrio cholerae</i>	≥ 40x	≥ 30	3800000-4300000	≥ 70	≥ 95	≤ 1	≥ 95	≤ 3	≥ 135 (300c) ≥ 225 (500c) ≥ 270 (600c)
<i>Vibrio parahaemolyticus</i>	≥ 40x	≥ 30	4900000-5500000	≥ 70	≥ 95	≤ 1	≥ 95	≤ 3	≥ 135 (300c) ≥ 225 (500c) ≥ 270 (600c)
<i>Vibrio vulnificus</i>	≥ 40x	≥ 30	4700000-5300000	≥ 70	≥ 95	≤ 1	NA ³	≤ 3	≥ 135 (300c) ≥ 225 (500c) ≥ 270 (600c)
<i>Cronobacter</i> spp.	≥ 40x	≥ 30	4000000-4900000	≥ 70	≥ 92	≤ 1	≥ 85	≤ 3	≥ 135 (300c) ≥ 225 (500c) ≥ 270 (600c)
<i>Yersinia</i> spp.	≥ 40x	≥ 30	4400000-5400000	≥ 70	≥ 92	≤ 1	≥ 85 ⁴	≤ 3	≥ 135 (300c) ≥ 225 (500c) ≥ 270 (600c)

¹ The pipeline will not further process the fastq files if the trimmed average read coverage (the coverage after quality-based trimming but before assembly) <10x.

² Core Genome Multi-Locus Sequence Typing (cgMLST) scheme is applicable to *C. jejuni* only.

³ NA, Not Applicable, core schemes available only for *V. cholerae* and *V. parahaemolyticus* (the whole genome scheme can be used for *V. vulnificus*).

⁴ Core Genome Multi-Locus Sequence Typing (cgMLST) scheme is applicable to *Y. enterocolitica* only

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Appendix PNQ09-4. Local Cluster Detection Thresholds for Detecting WGS Clusters

- **cgMLST** is the primary cluster detection method with the exception of *Listeria* and *Vibrio* species where **wgMLST** is the primary cluster detection method.
- ***Campylobacter*, *Escherichia*, *Shigella* spp., and *Salmonella*:** Identify local clusters of three or more human cases with no more than 10 cgMLST allele differences. At least two cases should have no more than 5 alleles of difference.
 - Exception for **clonal serotypes**: 3 or more human cases with no more than 5 allele differences for serotypes Newport, Javiana and Typhi, as well as *Shigella* species, and no more than 3 allele differences for serotype Enteritidis.
 - Collection dates should be within the last 60 days prior to detection, and PulseNet upload dates should also be within this 60-day timeframe.
- ***Listeria*:** Look for local clusters of sequences of three or more human cases within 25 wgMLST allele differences, with two being no more 10 alleles (wgMLST) different.
 - Alternatively, look for local clusters of sequences representing three or more human cases within 10 cgMLST allele differences with two being no more than 5 alleles different.
 - Collection dates should be within the past 120 days prior to detection. PulseNet upload dates should be within this 120-day timeframe.
- ***Vibrio*:** Look for local clusters of sequences representing three or more human cases within 15 wgMLST allele differences.
 - Collection dates should be within the past 60 days prior to detection. PulseNet upload dates should be within this 60-day timeframe.

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Appendix PNQ09-5. Proficiency Testing Cover Letter Template



Month yyyy

Dear *Participant(s), Primary PulseNet Contact, and Lab Director,*

The *yyyy* round of WGS PulseNet Proficiency Testing that included *Organism(s)* has been completed. The results for your laboratory are enclosed.

Below is the breakdown of the number of laboratories who have passed this round:

19/21 laboratories passed 2 are currently pending*

*Laboratories that did not pass were notified in *month* along with a request for resubmission.

A minimum 85% of all points available was required to pass this proficiency testing round. A thorough description of the scoring system is included in each report, as well as specific comments and suggestions for your laboratory, when applicable. Detailed summary reports are posted in PulseNet Announcements on SharePoint. PulseNet laboratory staff should have access to this forum. If your laboratory also participates in GenomeTrakr, your report has been shared with the GenomeTrakr PT contact(s).

Successful completion of the annual Proficiency Testing maintains your standing as a certified PulseNet laboratory and satisfies the annual PT requirements set forth by GenomeTrakr. If you have any questions, please send an email to PulseNet@cdc.gov. We appreciate your participation and continued support of PulseNet.

Sincerely,

name
contact info

Cc: PulseNet Team, Centers for Disease Control and Prevention
Food Safety Team, Association of Public Health Laboratories

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**Appendix PNQ09-6. Proficiency Testing Notification of Repeated Failure and
Loss of Certification Template**

[Save on CDC Letterhead]

[Date]

[Name and info of lab director],

The *[laboratory]* PulseNet laboratory failed the *[yyyy]* round of the PulseNet WGS Proficiency Testing three times. *[Insert reason for fail]*. The failure to pass Proficiency Test despite three attempts will result in the loss of the laboratory's fastq certification. The *[laboratory]* laboratory must resubmit the fastq certification panel in order to reinstate their certification status and the privilege to submit data to the national databases.

[Person] was notified on *[date]* and a fastq certification resubmission was requested. Comments and suggestions for improvement were provided. PulseNet participants are always reminded to contact their designated PulseNet Area Laboratory or CDC with any questions or for troubleshooting assistance.

Please let us know if you have any questions or concerns.

Thank you,

Name

Contact info