

LABORATORY STANDARD OPERATING PROCEDURE FOR PULSENET TOTAL DNA EXTRACTION AND QUALITY CONTROL OF PURIFIED DNA EXTRACTS			
Doc. No. PNL33	Ver. No. 01	Effective Date:	Page 1 of 21

1. **PURPOSE:** To describe the primary standard operating procedure (SOP) for manual total DNA extractions from pure single colony enteric bacterial isolates by Qiagen DNeasy Blood & Tissue kit and quality control of purified extracts by Nanodrop and Qubit for purposes of whole genome sequencing (WGS). This SOP facilitates inter-laboratory comparability of sequencing results.

2. **SCOPE:** For use by PulseNet participants when performing manual DNA extractions from enteric organisms for subsequent submission of WGS data to PulseNet and NCBI. Laboratories may amend this procedure as necessary after validation or use an alternative method (automated DNA extraction method) according to their laboratory's guidelines, following the minimum requirements of DNA quality and quantity stated in this SOP.

3. **DEFINITIONS:**
 - 3.1. **AE:** Elution Buffer
 - 3.2. **AL:** Lysis Buffer
 - 3.3. **ATL:** Tissue Lysis Buffer
 - 3.4. **AW1:** Wash Buffer 1
 - 3.5. **AW2:** Wash Buffer 2
 - 3.6. **BR:** Broad Range
 - 3.7. **BSC:** Biosafety Cabinet
 - 3.8. **BSL2:** Biosafety Level 2
 - 3.9. **DNA:** Deoxyribonucleic Acid
 - 3.10. **DNase:** Deoxyribonuclease
 - 3.11. **dsDNA:** Double-Stranded DNA
 - 3.12. **EDTA:** Ethylenediaminetetraacetic Acid
 - 3.13. **GHS:** Globally Harmonized System
 - 3.14. **HS:** High Sensitivity
 - 3.15. **NGS:** Next Generation Sequencing
 - 3.16. **NaOCl:** Sodium Hypochlorite (Bleach)
 - 3.17. **NaOH:** Sodium Hydroxide
 - 3.18. **NCBI:** National Center for Biotechnology Information
 - 3.19. **PCR:** Polymerase Chain Reaction
 - 3.20. **PHL:** Public Health Laboratory
 - 3.21. **PPE:** Personal Protective Equipment
 - 3.22. **QC:** Quality Control
 - 3.23. **RNase:** Ribonuclease
 - 3.24. **SDS:** Safety Data Sheet
 - 3.25. **SOP:** Standard Operating Procedure
 - 3.26. **Tris-HCl:** Tris Hydrochloride
 - 3.27. **TSA-SB:** Trypticase Soy Agar with 5% Sheep Blood

LABORATORY STANDARD OPERATING PROCEDURE FOR PULSENET TOTAL DNA EXTRACTION AND QUALITY CONTROL OF PURIFIED DNA EXTRACTS			
Doc. No. PNL33	Ver. No. 01	Effective Date:	Page 2 of 21

3.28. **WGS:** Whole Genome Sequencing

4. **RESPONSIBILITIES:**

4.1. **PulseNet Public Health Laboratory:**

4.1.1. Perform purified DNA extractions and quality control from pure single colony enteric bacterial isolates for WGS as described in this SOP.

4.1.2. Communicate with PulseNet Central as necessary for assistance with this SOP, suspected reagent issues and/or suspected instrument issues.

4.2. **PulseNet Central:**

4.2.1. Perform purified DNA extractions and quality control from pure single colony enteric bacterial isolates for WGS as described in this SOP.

4.2.2. Communicate any suspected reagent issues to PHLs as necessary.

4.2.3. Amend this SOP with any further changes, notify the PHLs, and update the SOP on SharePoint site.

5. **SAFETY:**

5.1. **Biosafety Warning:** Foodborne pathogens are capable of causing serious disease. Always use a minimum of BSL2 practices, and use extreme caution when transferring and handling strains of this type. Wear appropriate PPE when handling infectious organisms. Work in a BSC when handling organisms with a low infectious dose. Disinfect or dispose of all plastic ware and glassware that are exposed to the bacterial cultures.

5.2. **Chemical Safety Warning:** Take the required precautions, and wear appropriate PPE when handling potentially hazardous chemicals. Ensure that chemicals, spent containers, and unused contents are disposed of in accordance with governmental safety standards.

5.2.1. RNase A has a GHS Category 1 classification as a skin and respiratory sensitization reagent. See the SDS for more information.

5.2.2. Qiagen DNeasy Kit Components: See Qiagen's SDS for additional information.

5.2.2.1. **AL Buffer:** GHS Category 2 for skin & eye irritant. GHS Category 1 for skin sensitization. Contains a chaotropic salt and is not compatible with agents containing bleach.

5.2.2.2. **AW1 Buffer:** GHS Category 4 for oral & inhalation acute toxicity. GHS Category 2 for skin & eye irritation. Contains a chaotropic salt and is not compatible with agents containing bleach.

5.2.2.3. **Proteinase K:** GHS Category 1 for respiratory sensitization.

6. **SUPPLIES, REAGENTS, AND EQUIPMENT**

6.1. **DNA Extraction Reagents, Supplies and Equipment**

6.1.1. **Reagents:** All reagents are stored at 20-25°C unless noted otherwise. Manufacturer's expiration guidelines are followed unless noted otherwise.

**LABORATORY STANDARD OPERATING PROCEDURE FOR PULSENET TOTAL DNA
EXTRACTION AND QUALITY CONTROL OF PURIFIED DNA EXTRACTS**

Doc. No. PNL33

Ver. No. 01

Effective Date:

Page 3 of 21

- 6.1.1.1. TSA-SB or comparable media (ThermoFisher, Cat# R01198 or equivalent). Store at 4-8°C.
- 6.1.1.2. Ethanol, molecular-grade 95-100% (Fisher Scientific, Cat# BP2818-500 or equivalent)
- 6.1.1.3. Isopropanol, 99-100% (Sigma-Aldrich, Cat# I9516-500ML or equivalent)
- 6.1.1.4. RNase A, 100mg/ml (Qiagen, Cat# 19101 or equivalent) See Section 5.2.2 for safety information.
- 6.1.1.5. DNase free, molecular-grade water (Fisher Scientific, Cat# BP24701 or equivalent. Millipore-filtered water is also acceptable)
- 6.1.1.6. Tris-HCL, 1M, pH 8.0 (Sigma-Aldrich, Cat# T3038-1L or equivalent)
- 6.1.1.7. Lysozyme (Sigma-Aldrich, Cat# L4919 or equivalent), store at -20°C.
- 6.1.1.8. EDTA, 0.5M (Sigma-Aldrich, Cat# 03690-100 ml)
- 6.1.1.9. Triton-X 100 (Fisher Scientific, Cat# BP151-100)
- 6.1.1.10. Qiagen DNeasy Blood & Tissue Kit (Qiagen Cat# 69504 or 69506)
 - 6.1.1.10.1. AL Buffer (see Section 5.2.2.1 for safety information)
 - 6.1.1.10.2. ATL Buffer
 - 6.1.1.10.3. Proteinase K (see Section 5.2.2.3 for safety information)
 - 6.1.1.10.4. AWI Buffer (see Section 5.2.2.2 for safety information)
 - 6.1.1.10.5. AW2 Buffer
 - 6.1.1.10.6. Collection tubes
 - 6.1.1.10.7. Spin columns
- 6.1.1.11. Qubit dsDNA BR Assay Kit (ThermoFisher Cat# Q32850 or Q32853)
 - 6.1.1.11.1. dsDNA BR Reagent (Component A). Protect from light.
 - 6.1.1.11.2. dsDNA BR Buffer (Component B).
 - 6.1.1.11.3. dsDNA BR Standard #1 (Component C). Store at 2-8°C
 - 6.1.1.11.4. dsDNA BR Standard #2 (Component D). Store at 2-8°C
- 6.1.1.12. **OPTIONAL:** Genomic DNA Clean & Concentrator-5 Kit (Zymo Research, Cat# D4066)
- 6.1.1.13. **OPTIONAL:** Promega Wizard® Genomic DNA Purification Kit (Promega, Cat# A1120)

6.1.2. Supplies

- 6.1.2.1. 1 µl inoculation loops (ThermoFisher Cat# 254410 or equivalent)
- 6.1.2.2. 1.5 ml microcentrifuge tubes (ThermoFisher Cat# AM12400 or equivalent)
Note: Autoclave these tubes for sterilization before use.
- 6.1.2.3. 5 ml tubes (Fisher Scientific Cat# 14-959A or equivalent)
- 6.1.2.4. Pipette tips, filtered: 10 µl, 20 µl, 200 µl and 1000 µl volumes (Rainin Cat# 17001865, 17001863 & 17001864 or equivalent)

LABORATORY STANDARD OPERATING PROCEDURE FOR PULSENET TOTAL DNA EXTRACTION AND QUALITY CONTROL OF PURIFIED DNA EXTRACTS			
Doc. No. PNL33	Ver. No. 01	Effective Date:	Page 4 of 21

6.1.2.5. Qubit Assay Tubes [ThermoFisher Cat# Q32856 or equivalent (clear, thin-wall 0.5-ml PCR tubes)].

6.1.3. Equipment

6.1.3.1. Incubator (35-37°C)

6.1.3.2. Vortex

6.1.3.3. Water bath(s) or thermal block(s) accommodating 1.5 ml microcentrifuge tubes

6.1.3.4. Centrifuge, accommodating 1.5-2 ml tubes and up to 13,000-14,000 rpm

6.1.3.5. Micropipettes, single channel: 10 µl, 20 µl, 100 µl, 200 µl, 1000 µl volumes

6.1.3.6. Qubit 2.0 or 3.0 Fluorometer or equivalent for quantification of dsDNA

6.1.3.7. NanoDrop 2000 UV-Vis/Nanodrop Onespectrometer or equivalent for determination of 260/280 nm readings.

7. PROCEDURE

7.1. **Day 0 - Isolate preparation:** Isolated colonies must be obtained for sequencing. Prepare such colonies by streaking a plate for isolation from the original slant per your laboratory's methods. Use this plate with isolated colonies for the method below.

Note: *Minimize the number of passages from the slant to the plate for DNA extraction.*

7.1.1. Streak an isolated colony from a pure plate to a TSA-SB plate (or comparable media) and incubate at 37°C ± 1 for 16-24 hours.

7.2. Day 1 - DNA extraction using the Qiagen DNeasy Blood & Tissue Kit

NOTE 1: *If using the Promega Wizard DNA extraction procedure, see Appendix PNL33-1. A minimum of 4 - 6 hours must be allocated to complete the process using this kit.*

NOTE 2: *DNA extractions to be used for next generation library preparation and sequencing must not contain EDTA. This chemical has been found to inhibit certain processes during library prep (i.e. tagmentation) and is found in the elution / rehydration buffers of many commercial kits. EDTA inactivates enzymes and/or buffers by chelation with metal ions found in these reagents. It is for this reason that 10 mM Tris-HCL, pH 8.0 is used as an elution buffer instead of the elution buffer included in the extraction kit.*

7.2.1. Before beginning this procedure, first:

7.2.1.1. Pre-heat water bath or heat block to 56°C ± 1.

7.2.1.2. Check that the DNeasy kit reagents have been prepared. If not, dilute with ethanol (molecular-grade 95-100%) where necessary, following the kit insert protocol. Initial and date the bottles after dilution for purposes of record-keeping and to indicate that the reagents are ready for use.

7.2.1.3. Prepare the elution buffer, 10 mM Tris HCl pH 8.0, as follows:

LABORATORY STANDARD OPERATING PROCEDURE FOR PULSENET TOTAL DNA EXTRACTION AND QUALITY CONTROL OF PURIFIED DNA EXTRACTS

Doc. No. PNL33

Ver. No. 01

Effective Date:

Page 5 of 21

- 7.2.1.3.1. Add 10 ml of 1 M Tris-HCl, pH 8.0 to 990 ml molecular-grade water.
- 7.2.1.3.2. Invert several times to mix.
- 7.2.1.3.3. Store at 2-8°C for up to one year (indicate the initial, date of preparation and date of expiry on the bottle).

7.2.2. Cell lysis of Gram-positive organisms:

7.2.2.1. Prepare the enzymatic lysis buffer (ELB), comprised of 20 mM Tris-HCl, 2 mM EDTA, 1.2 % Triton:

7.2.2.1.1. Combine the following:

5 ml 1 M Tris-HCl, pH 8.0

1 ml 0.5 M EDTA

3 ml Triton X-100

241 ml molecular-grade water

7.2.2.1.2. Mix gently to incorporate Triton X (this may be done with a stir bar and a stir plate if preferred). Store at room temperature for up to one year.

7.2.2.2. Prepare ELB-lysozyme solution by adding lysozyme to the appropriate volume of ELB to achieve a 20 mg/ml lysozyme concentration. The chart below can be used as a guide. Thoroughly mix the ELB-Lysozyme solution prior to dispensing.

NOTE1: *Increased efficacy has been observed after allowing the ELB-Lysozyme solution incubate at room temperature for 20-30 minutes.*

NOTE2: *The solution must be used within 24 hours of preparation.*

Num. of samples + 1 extra	Total ELB volume (µl)	Lysozyme (mg)
(5+1) = 6	1080	21.6
(10+1) = 11	1980	39.6
(18+1) = 19	3420	68.4
(20+1) = 21	3780	75.6

Table 1: Total volume of ELB and lysozyme needed for different number of samples

- 7.2.2.3. Dispense 180 µl of prepared ELB-lysozyme mixture to each labeled 1.5 ml microcentrifuge tube.
- 7.2.2.4. Fill a 1 µl inoculating loop with overnight growth (see image below), add this to the 180 µl ELB-lysozyme mixture in 1.5 ml sterile microcentrifuge tube. Vortex at high speed for approximately 5-10 seconds.

**LABORATORY STANDARD OPERATING PROCEDURE FOR PULSENET TOTAL DNA
EXTRACTION AND QUALITY CONTROL OF PURIFIED DNA EXTRACTS**

Doc. No. PNL33

Ver. No. 01

Effective Date:

Page 6 of 21



Figure 1: The recommended amount of overnight bacterial culture in a 1 µl inoculating loop for DNA extraction

7.2.2.5. Incubate at $56^{\circ}\text{C} \pm 1$ for 30 minutes.

NOTE: *Vortexing every 10 minutes throughout the incubation process is recommended in order to improve cell lysis.*

7.2.2.6. Add 4 µl of RNase A solution and vortex at medium speed (e.g. speed '7' on a Daigger Vortex Genie) for 5-10 seconds.

7.2.2.7. Incubate at room temperature for 3-5 minutes.

7.2.2.8. Add 25 µl Proteinase K and 200 µl AL buffer, and vortex for 5-10 seconds at medium speed.

NOTE: *Vortex immediately after the addition of buffer AL to each sample for increased DNA yield. It is NOT recommended to vortex until after the addition of buffer AL to all samples.*

7.2.2.9. Incubate at $56^{\circ}\text{C} \pm 1$ for 30 minutes.

7.2.2.10. Add 200 µl of 95-100% ethanol and vortex for 5-10 seconds at medium speed. Proceed to section 7.2.4.

NOTE: *A clear or white precipitate may form on addition of ethanol. It is essential to apply all of the precipitate to the DNeasy spin column in the next step (Section 7.2.4.1.).*

7.2.3. Cell lysis of Gram-negative organisms:

7.2.3.1. Dispense 180 µl of buffer ATL to labeled 1.5 ml sterile microcentrifuge tubes.

7.2.3.2. Fill a 1 µl inoculating loop with overnight growth (Figure 1), add this to 180 µl buffer ATL in a 1.5 ml microcentrifuge tube, and vortex at high speed for approximately 5-10 seconds.

7.2.3.3. Add 20 µl Proteinase K to each sample tube and vortex at high speed for 5-10 seconds.

7.2.3.4. Incubate at $56^{\circ}\text{C} \pm 1$ for 1-3 hours, vortexing every 20 minutes throughout the incubation process to improve cell lysis.

LABORATORY STANDARD OPERATING PROCEDURE FOR PULSENET TOTAL DNA EXTRACTION AND QUALITY CONTROL OF PURIFIED DNA EXTRACTS			
Doc. No. PNL33	Ver. No. 01	Effective Date:	Page 7 of 21

7.2.3.5. Add 4 µl of RNase A solution and vortex at medium speed (e.g. '7' on a Daigger Vortex Genie) for 5-10 seconds.

7.2.3.6. Incubate at room temperature for 3-5 minutes.

7.2.3.7. Add 200 µl buffer AL and vortex for 5-10 seconds.

NOTE: *Vortex immediately after the addition of buffer AL to each sample for increased DNA yield. It is NOT recommended to vortex until after the addition of buffer AL to all samples.*

7.2.3.8. Add 200 µl of 95% – 100% ethanol and vortex 5-10 seconds.

NOTE: *A clear or white precipitate may form on addition of buffer AL. It is essential to apply all of the precipitate to the DNeasy spin column in the next step.*

7.2.4. DNA clean-up and elution:

7.2.4.1. Pour the mixture into a DNeasy spin column set in a 2 ml collection tube.

NOTE: *Ensure that mixture is well suspended prior to transferring to the spin column.*

7.2.4.2. Centrifuge at 10,000 rpm for 1 minute and discard the flow-through and collection tube.

7.2.4.3. Place the DNeasy spin column in a new 2 ml collection tube and add 500 µl buffer AW1 (invert buffer several times to mix before use).

7.2.4.4. Centrifuge at 10,000 rpm for 1 minute and discard the flow-through and collection tube.

7.2.4.5. Place the DNeasy spin column in a new 2 ml collection tube and add 500 µl Buffer AW2 (invert buffer several times to mix before use).

7.2.4.6. Centrifuge at 13,000 – 14,000 rpm for 3 minutes to collect the flow-through in the collection tube and dry the membrane. Discard the flow-through and collection tube.

OPTIONAL: *During this step, prepare a tube with enough 10 mM Tris-HCl pH 8.0 for 100 µl per sample + 1 extra, and incubate at 56°C ± 1°C while samples are centrifuging. This is done to bring the elution buffer (10 mM TRIS-HCl pH 8.0 to an optimal elution temperature.*

7.2.4.7. Place the DNeasy spin column in a clean, labeled 1.5 ml microcentrifuge tube and add 100 µl of pre-warmed 10 mM Tris-HCl pH 8.0 directly onto the DNeasy membrane. Be sure not to touch the membrane with the pipette tip.

7.2.4.8. Incubate at room temperature for 3 – 5 min.

7.2.4.9. Centrifuge at 10,000 – 11,000 rpm for 1 minute to collect the eluate in the new tube.

7.2.4.10. DNA may be checked for quality and quantity at this time. If DNA is to be sequenced within 1 week of extraction it may be stored at 2 – 8°C. For long term storage, store at -15 to -80°C and avoid repeated freeze-thaws.

7.3. DNA Quality Control

7.3.1. Determination of DNA quality using the Nanodrop 2000 Spectrophotometer

7.3.1.1. Select Nanodrop 2000 icon on the desktop of the Nanodrop workstation.

7.3.1.2. Select appropriate application from the screen, in this case “nucleic acid”, and follow prompts to begin initialization.

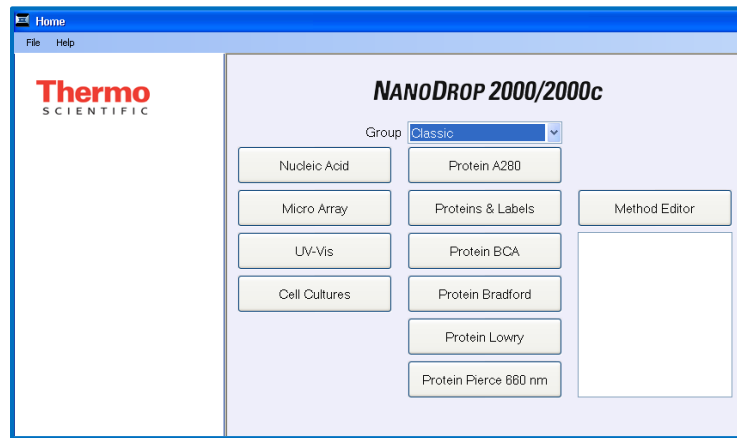


Figure 2: A screen shot of NanoDrop 2000 operation

When asked if you would like to open up your last workbook, choose “yes” if you would like to append results to previously saved data, or choose “no” if you would like to begin a new workbook.

NOTE: *The arm of the Nanodrop must be lowered during initialization.*

7.3.1.3. Lift the arm of the Nanodrop and use a lint-free laboratory wipe dampened with distilled H₂O to wipe down the top and bottom pedestals.

7.3.1.4. Pipette 1-2 µl of blanking solution (e.g. 10 mM Tris-HCl pH 8.0) onto the bottom pedestal and lower the arm after loading. Ensure that “Add to report” is selected in the upper left quadrant of the screen so that measurements are saved, and click the “Blank” button on the screen (upper left).

NOTE: *Ensure that there are no bubbles in the sample on the stand so that an accurate read is obtained.*

LABORATORY STANDARD OPERATING PROCEDURE FOR PULSENET TOTAL DNA EXTRACTION AND QUALITY CONTROL OF PURIFIED DNA EXTRACTS

Doc. No. PNL33

Ver. No. 01

Effective Date:

Page 9 of 21

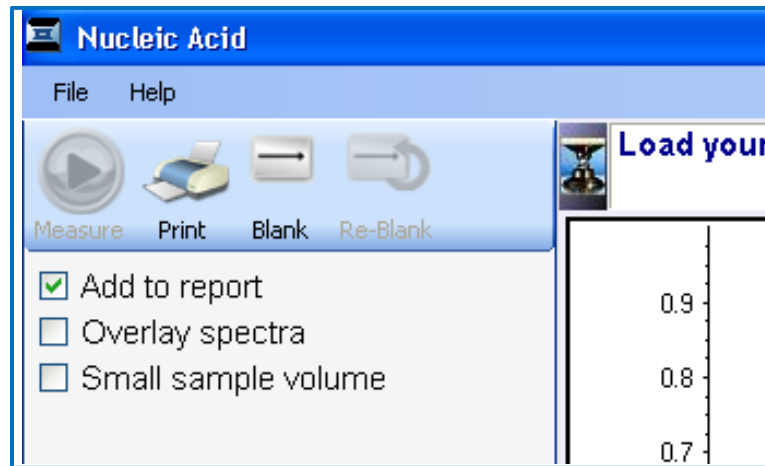


Figure 3: A screen shot of NanoDrop 2000 operation (continuation)

- 7.3.1.5. After the instrument has read the blank, lift the arm and wipe both pedestal and arm with a dry laboratory wipe to ready the Nanodrop for measuring samples.
- 7.3.1.6. Pipette 1-2 μ l of undiluted DNA sample onto the bottom pedestal and lower the arm.
- 7.3.1.7. Enter the sample name in the “Sample ID” field (upper right) and click “Measure” (upper left). When the measurement has been taken, results will populate the screen.
- 7.3.1.8. A prompt will appear after the first sample is measured: follow the prompt to save the workbook. The workbook must be saved before continuing.
- 7.3.1.9. Wipe both pedestals clean with a lint-free wipe dampened with deionized water and repeat steps 7.3.1.6 – 7.3.1.8 for all samples.
- 7.3.1.10. After all samples have been measured, click on “Reports” icon in the lower left quadrant of the screen to print and/or export data if desired.
- 7.3.1.11. Record the data by either electronic export as described below or by manual recording.
 - 7.3.1.11.1. Click on the “Reports” icon in the lower left quadrant of the screen.
 - 7.3.1.11.2. Select “Export”.
 - 7.3.1.11.3. Save the NanoDrop workbook to the appropriate EDLB network space or to a flash drive.
- 7.3.1.12. Transfer the data into the appropriate log sheet or WGS workbook.
- 7.3.1.13. Readings for 260/280 field should be between **1.75 and 2.05 (this includes a 0.05 or 3% error rate)** for the extracted DNA to be accepted as ‘pure’ and suitable for WGS. If the ratio is lower than 1.75, it may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm.

LABORATORY STANDARD OPERATING PROCEDURE FOR PULSENET TOTAL DNA EXTRACTION AND QUALITY CONTROL OF PURIFIED DNA EXTRACTS			
Doc. No. PNL33	Ver. No. 01	Effective Date:	Page 10 of 21

NOTE1: *If 260/280 values are consistently within range over the course of several runs, then it is not necessary to routinely read the values on the Nanodrop for subsequent extractions.*

NOTE2: *if the 260/280 values are outside the acceptable range, the DNA extract can be purified by using the Zymo DNA clean-up and concentrator kit (see Appendix PNL33-2 for instructions).*

7.3.2. Determination of DNA quality using the Nanodrop One Spectrophotometer

7.3.2.1. Touch the screen to activate the instrument.

7.3.2.2. Select the “Nucleic Acids” tab on the Home screen.

7.3.2.3. Select “dsDNA”.

7.3.2.4. Lift the instrument arm and clean the upper and lower pedestals with a new lint-free laboratory wipe moistened with distilled H₂O.

7.3.2.5. Pipet 1-2 µl blanking solution onto the lower pedestal and lower the arm.

7.3.2.6. Allow the instrument to perform the auto-blank step.

NOTE: *The auto-blank feature has been turned on for this instrument. Do not change this setting. If auto-blank has been turned off, tap “Blank” and allow the measurement to complete.*

7.3.2.7. Lift the arm and clean both pedestals with a lint-free laboratory wipe.

7.3.2.8. Tap the Sample Name field to display the keyboard.

7.3.2.9. Enter the Sample Name.

7.3.2.10. Tap done.

7.3.2.11. Pipet 1-2 µl of DNA sample onto the pedestal and lower the arm.

7.3.2.12. Allow the instrument to perform the Auto-Measure step.

NOTE: *The auto-measure feature has been turned on for this instrument. Do not change this setting. If auto-measure has been turned off, tap “Measure” and allow the measurement to complete.*

7.3.2.13. Repeat steps 7.3.2.7 - 7.3.2.12 until all samples have been measured.

7.3.2.14. Tap “End Experiment” after all samples have been measured.

7.3.2.15. Lift the arm and clean both pedestals with a clean lint-free wipe moistened with distilled H₂O.

7.3.2.16. Record the data by either electronic export as described below or by manual recording.

NOTE: *The following steps detail exporting of data immediately at the end of an experiment. Refer to the NanoDrop One Help User Guide for instructions on saving an experiment for export at a later time.*

7.3.2.16.1. Insert a flash drive into the USB port.

7.3.2.16.2. Tap “Export” and select the .csv file format.

7.3.2.16.3. Tap “Export” again.

LABORATORY STANDARD OPERATING PROCEDURE FOR PULSENET TOTAL DNA EXTRACTION AND QUALITY CONTROL OF PURIFIED DNA EXTRACTS

Doc. No. PNL33

Ver. No. 01

Effective Date:

Page 11 of 21

7.3.2.16.4. Tap OK after the export success message appears.

7.3.2.16.5. Remove the flash drive.

7.3.2.16.6. Tap “End Experiment”.

7.3.2.17. Transfer the data into the appropriate log sheet, worksheet, or WGS workbook.

7.3.3. Determination of DNA quantification using Qubit 2.0 or 3.0 Fluorometer

NOTE: *The Broad Range (BR) kit is designed to be accurate for initial sample concentrations from 100 pg/μl to 1,000 ng/μl. Use for quantitation of product containing higher concentrations of DNA (i.e. gDNA extractions). The High Sensitivity Kit (HS) is designed to be accurate for initial sample concentrations from 10 pg/μl to 100 ng/μl. Use for quantitation of product containing a lower concentration of DNA (i.e. PCR product).*

7.3.3.1. Set up and label one assay tube for each sample and the two standards.

7.3.3.2. Prepare the Qubit working solution using a 1:200 ratio of Qubit Reagent to Qubit Buffer for each sample and the two standards. See table for an example of calculations.

NOTE: *Store the HS / BR Reagent away from light exposure, as it contains fluorescent components.*

Reagents	1x	Example: 8 samples + 2 standards
Qubit HS or BR Reagent	1 μl	= 10 μl
Qubit HS or BR Buffer	199 μl	= 1990 μl
Total volume (Working Solution)	200 μl	= 2000 μl

Table 2: Total volume of Qubit reagent and buffer needed to prepare working solution for 8 samples and 2 standards

NOTE: *It is recommended to increase the number of samples used in the calculation by at least one to ensure sufficient volume.*

7.3.3.3. For each standard, dispense 190μl of working solution and add 10 μl of the Qubit standards into the respective assay tubes.

7.3.3.4. Dispense 198μl Working Solution and 2μl of input DNA to each sample assay tube. Final volume for each sample and standard should be 200 μl.

7.3.3.5. Vortex all tubes for 2 – 3 seconds.

LABORATORY STANDARD OPERATING PROCEDURE FOR PULSENET TOTAL DNA EXTRACTION AND QUALITY CONTROL OF PURIFIED DNA EXTRACTS			
Doc. No. PNL33	Ver. No. 01	Effective Date:	Page 12 of 21

- 7.3.3.6. Incubate all tubes for a minimum of 2 minutes at room temperature and read within 1 hr. The short time frame is required to minimize the exposure of the reagent to light.

NOTE 1: *Ensure that the assay is performed entirely at room temperature. Do not heat / hold the assay tubes in your hand prior to measurement, since warming the solution results in lower readings.*

NOTE 2: *The temperature inside the Qubit® Fluorometer may be as much as 3°C above room temperature after 1 hour. For this reason, if you want to perform multiple readings of a single tube, remove the tube from the instrument and let it equilibrate to room temperature for 30 seconds before taking another reading.*

7.3.4. Qubit 2.0 Fluorometer Operation

- 7.3.4.1. Touch the screen to activate the device.
- 7.3.4.2. When prompted to choose the sample type, select “DNA.”
- 7.3.4.3. When prompted to choose the assay type select “dsDNA Broad Range” or “dsDNA High Sensitivity” depending on the assay used.
- 7.3.4.4. When asked whether new standards are available, select “yes”.
- 7.3.4.5. Insert Standard 1 and push “Read.”
- 7.3.4.6. Insert Standard 2 and push “Read.”

NOTE: *If the instrument is unable to read the standards, start the process over by preparing new standards and samples using the instructions outlined in step 7.3.3.*

- 7.3.4.7. Insert a sample tube into the sample chamber, close the lid, and push “Read.”
- 7.3.4.8. Once the initial read is complete, push “Calculate Stock Concentration.”
- 7.3.4.9. Select the sample volume (1-20 µL) on the volume roller wheel and the desired units from the drop-down menu (tap on the displayed units to prompt).

NOTE: *The units can be converted to ng/µl (or another unit) by pushing the button displaying the units (next to the concentration read out) and selecting the desired unit in the pop-up window (see below).*

LABORATORY STANDARD OPERATING PROCEDURE FOR PULSENET TOTAL DNA EXTRACTION AND QUALITY CONTROL OF PURIFIED DNA EXTRACTS

Doc. No. PNL33

Ver. No. 01

Effective Date:

Page 13 of 21

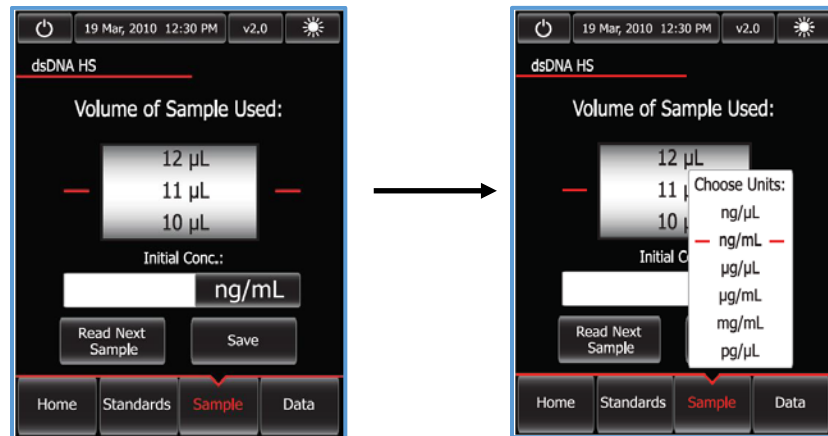


Figure 4: A screenshot of the operation of the Qubit 2.0 Fluorometer for selection of sample volume and unit

- 7.3.4.10. Push “Save.” A progress bar will appear indicating that the displayed data is being saved to the instrument.
NOTE: *The “Save” button can be pushed multiple times without creating duplicate entries.*
- 7.3.4.11. Proceed to read the next sample by swapping the tubes, selecting “Read Next Sample,” and repeating steps 7.3.4.7 – 7.3.2.10 until all samples have been read.
- 7.3.4.12. When all samples have been read, push the “data” button at the bottom right of the screen.
- 7.3.4.13. Insert the flash drive in the designated slot. Highlight the readings for the samples of interest and push the flash drive icon on the lower left of the screen to transfer the data. A progress bar will be displayed to show the status of the process.
NOTE: *A green dot on the USB icon indicates that the instrument recognizes the flash drive. If the flash drive is not present, not inserted properly, or cannot be recognized, a red dot will be displayed instead.*
- 7.3.4.14. Remove the flash drive. The saved .CSV file can now be opened in Microsoft Excel on any computer workstation.
- 7.3.4.15. Samples are listed, from top to bottom, **in the order of most recent reading to the initial reading.**
- 7.3.4.16. Rename the samples accordingly. The default sample names under the “Name” column when the file is opened in Excel will be the time at which each sample was read.
- 7.3.4.17. These DNA concentrations may be recorded on respective NGS library prep workbooks.

OPTIONAL: *DNA concentrations can also be written down in lieu of using the flash drive, primarily for situations where only a few samples have been quantitated.*

7.3.5. Qubit 3.0 Fluorometer Operation

7.3.5.1. Touch the screen to activate the device.

7.3.5.2. When prompted to choose the sample type select “dsDNA.”

7.3.5.3. When prompted to choose the assay type select “dsDNA Broad Range” or “dsDNA High Sensitivity” depending on the assay used.

7.3.5.4. Touch “read standards.”

7.3.5.5. At the prompt, insert standard 1 and select “read standard 1.”

7.3.5.6. At the prompt, insert standard 2 and select “read standard 2.”

NOTE: *If the instrument is unable to read the standards, start the process over by preparing new standards and samples using the instructions outlined in steps 7.3.3.*

7.3.5.7. Select “run samples.”

7.3.5.8. In the sample volume screen, select the sample volume (1-20 μ L) by touching the + or – buttons on the wheel.

7.3.5.9. Select the units for the output sample concentration from the pulldown menu.



Figure 5: A screenshot of the operation of the Qubit 3.0 Fluorometer for selection of sample volume and unit

7.3.5.10. Insert a sample tube into the sample chamber, close the lid, and select “read tube.”

7.3.5.11. Proceed to the next sample and repeat step 7.3.5.10 until all samples have been read.

LABORATORY STANDARD OPERATING PROCEDURE FOR PULSENET TOTAL DNA EXTRACTION AND QUALITY CONTROL OF PURIFIED DNA EXTRACTS			
Doc. No. PNL33	Ver. No. 01	Effective Date:	Page 15 of 21

7.3.5.12. Select “data.”

NOTE: *The blue chevron above the sample number reverses the order of the data entries.*

7.3.5.13. Insert the flash drive in the designated slot and select “export.”

7.3.5.14. Select the assay to be exported to the flash drive.

NOTE: *The screen will display all assays that have been saved onto the instrument in chronological order, the most recent will be at the top.*

7.3.5.15. Select “export.”

NOTE: *The export data screen displays the number of successful samples saved onto the flash drive.*

7.3.5.16. Select “done.”

7.3.5.17. Touch the home icon.

7.3.5.18. Remove the flash drive. The saved .CSV file can now be opened in Microsoft Excel on any computer workstation

7.3.5.19. Samples are listed, from top to bottom, **in the order of the most recently read to the first read.**

7.3.5.20. Rename the samples accordingly. The default sample names under the “Name” column when the file is opened in Excel will be the time at which each sample was read.

7.3.5.21. These DNA concentrations may be recorded on respective NGS library prep workbooks.

OPTIONAL: *DNA concentrations can also be written down in lieu of using the flash drive, primarily for situations where only a few samples have been quantitated.*

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LABORATORY STANDARD OPERATING PROCEDURE FOR PULSENET TOTAL DNA EXTRACTION AND QUALITY CONTROL OF PURIFIED DNA EXTRACTS			
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Doc. No. PNL33	Ver. No. 01	Effective Date:	Page 16 of 21
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9. AMENDMENTS:

9.1. **01/24/2019:**

- **Pulled DNA Extraction and Quality Control Procedures out of PNL32 to create separate document (PNL33).**

10. APPROVAL SIGNATURES:

Approved By: _____ Date: _____
PulseNet QA/QC Personnel

Approved By: _____ Date: _____
PulseNet Outbreak Detection and Surveillance Unit Chief

Approved By: _____ Date: _____
PulseNet PFGE Reference Unit Chief

Approved By: _____ Date: _____
PulseNet Next Generation Subtyping Methods Unit Chief

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

Appendix PNL33-1

DNA Extraction / template preparation using the Promega Wizard Genomic DNA Purification Kit

1. Procedure

1.1. Day 1

1.1.1. Streak an isolated colony from test cultures to TSA-SB plate (or comparable media).

Incubate cultures at $37-42^{\circ}\text{C} \pm 1$ for 16-24 hours.

NOTE: *If using culture from broth see step 1.2.1.1.2. for Gram-negative bacteria and step 1.2.2.4.2. for Gram-positive bacteria. The procedure will then start on Day 3.*

1.2. Day 2

1.2.1. Cell lysis for Gram-negative bacteria

1.2.1.1. Harvest bacterial culture:

1.2.1.1.1. Plated culture:

1.2.1.1.1.1. Dispense 600 μl of Nucleic Lysis Solution into sterile 1.5 ml microfuge tubes

1.2.1.1.1.2. Fill a 10 μl inoculating loop approximately one-fourth full of growth and add to the Nucleic Lysis Solution

1.2.1.1.2. Broth culture:

1.2.1.1.2.1. Add 1 ml of broth culture to a 1.5 ml microcentrifuge tube.

1.2.1.1.2.2. Centrifuge at 13,000-14,000 x g (10,000-11,000 rpm) to pellet cells. Remove and discard as much of the supernatant as possible without agitating cells.

1.2.1.1.2.3. Resuspend in 600 μl of Nuclei Lysis Solution.

1.2.1.2. Mix by vortexing for 5-10 seconds or by pipetting up and down until solution appears homogenized.

1.2.1.3. Incubate the sample at $80^{\circ}\text{C} \pm 1$ for 5 minutes to lyse the cells.

OPTIONAL: *Incubating at $60^{\circ}\text{C} \pm 1$ for 45 minutes (instead of $80^{\circ}\text{C} \pm 1$ for 5 minutes) may improve cell lysis.*

1.2.1.4. Cool the sample by incubating on ice for 1 minute or on bench for 5 minutes.

1.2.1.5. Add 3 μl RNase A Solution to the cell lysate, and mix by inverting the tube 25 times.

1.2.1.6. Incubate at $37^{\circ}\text{C} \pm 1$ for 15-60 minutes.

1.2.2. Cell lysis for Gram-positive bacteria

1.2.2.1. Dilute sufficient 50 mM EDTA, pH 8.0 for the number of extractions being performed.

1.2.2.1.1. 50 mM EDTA, pH 8.0 = 100 μl stock 0.5 M EDTA + 900 μl molecular-grade water. Mix by inverting several times and store at room temperature ($15-30^{\circ}\text{C}$)

LABORATORY STANDARD OPERATING PROCEDURE FOR PULSENET TOTAL DNA EXTRACTION AND QUALITY CONTROL OF PURIFIED DNA EXTRACTS

Doc. No. PNL33

Ver. No. 01

Effective Date:

Page 18 of 21

- 1.2.2.2. Prepare sufficient amount of 20 mg/μl lysozyme solution (Lytic Enzyme Solution) by adding lysozyme powder to the 50 mM EDTA, pH 8.0 according to the chart below.

NOTE: *Lysozyme may take up to 10 minutes to completely dissolve into solution.*

Number of Samples + 1	Total Volume 50 mM EDTA (μl)	Lysozyme (mg)
5 + 1 = 6	1080	21.6
10 + 1 = 11	1980	39.6
18 + 1 = 19	3420	68.4
20 + 1 = 21	3780	75.6

Table 1: Volume of EDTA and Lysozyme needed to prepare Lytic Enzyme Solution for various number of samples

- 1.2.2.3. Harvest bacterial culture:
- 1.2.2.3.1. Plated culture:
- 1.2.2.3.1.1. Dispense 420 μl of 50 mM EDTA, pH 8.0 into sterile 1.5 ml microfuge tubes
- 1.2.2.3.1.2. Fill a 10 μl inoculating loop approximately one-fourth full of growth and add to the 50 mM EDTA
- 1.2.2.3.2. Broth culture:
- 1.2.2.3.2.1. Add 1 ml of broth culture to a 1.5 ml microcentrifuge tube.
- 1.2.2.3.2.2. Centrifuge at 13,000-14,000 x g (10,000-11,000 rpm) to pellet cells. Remove and discard as much of the supernatant as possible.
- 1.2.2.3.2.3. Resuspend the cells in the 420 μl of 50 mM EDTA, pH 8.0.
- 1.2.2.4. Gently pipette and/or vortex until the cells are resuspended.
- 1.2.2.5. Dispense 180 μl of the Lytic Enzyme Solution to each sample and vortex to mix.
- 1.2.2.6. Incubate the sample at 56°C ± 1 for 60 minutes to lyse the outer cell wall.
- 1.2.2.7. Centrifuge the sample at 13,000-16,000 x g (10,000-11,000 rpm) for 1 minute. Remove and discard as much of the supernatant as possible.
- 1.2.2.8. Add 600 μl of Nuclei Lysis Solution. Gently pipette up and down until the cells are resuspended.
- 1.2.2.9. Incubate at 60°C ± 1 for 45 minutes to lyse the cells.
- 1.2.2.10. Cool the sample by incubating on ice for 1 minute or on the bench for 5 minutes.
- 1.2.2.11. Add 3 μl RNase A Solution, 4 mg/ml, to the cell lysate and mix by inverting the tube 25 times.
- 1.2.2.12. Incubate at 37°C ± 1 for 30-90 minutes.

LABORATORY STANDARD OPERATING PROCEDURE FOR PULSENET TOTAL DNA EXTRACTION AND QUALITY CONTROL OF PURIFIED DNA EXTRACTS			
Doc. No. PNL33	Ver. No. 01	Effective Date:	Page 19 of 21

1.2.3. Protein precipitation

- 1.2.3.1. Cool the sample by placing on ice for 1 minute or on the bench for 5 minutes.
- 1.2.3.2. Add 200 µl of Protein Precipitation Solution to the RNase-treated cell lysate.
- 1.2.3.3. Vortex vigorously at high speed for 20 seconds to mix.
- 1.2.3.4. Incubate the sample on ice for 10 minutes.
- 1.2.3.5. Centrifuge at 13,000-16,000 x g (10,000-13,000 rpm) for 3 minutes. The precipitated protein should form a tight pellet.

OPTIONAL: *If the protein did not form a tight pellet, repeat the above steps by pouring the supernatant containing the DNA (leaving behind the precipitated protein pellet) into a clean 1.5ml microcentrifuge tube, incubating the sample on ice for 5 minutes, and centrifuging at 13,000-16,000 x g or 10,000 – 13,000 rpm for 3 minutes.*

1.2.4. DNA Precipitation

- 1.2.4.1. Transfer the supernatant containing the DNA into a clean 1.5 ml microfuge tube containing 600 µl of 100% Isopropanol (2-propanol).
- 1.2.4.2. Mix the sample by gently inverting approximately 50 times.
- 1.2.4.3. Centrifuge at 13,000-16,000 x g (10,000-13,000 rpm) for 3 minutes. The DNA pellet should be visible as a small white pellet.
- 1.2.4.4. Pour off the supernatant. The pellet may be loose so pour slowly so as not to disturb the pellet.
- 1.2.4.5. Add 600 µl 70% Ethanol and invert the tube several times to wash the pellet.
- 1.2.4.6. Centrifuge at 13,000-16,000 x g (10,000-13,000 rpm) for 1-3 minutes. Carefully pour off the ethanol. Pour slowly as the pellet may be loose.
- 1.2.4.7. Briefly spin down the tube to collect any remaining ethanol to the bottom of the tube. Remove the remaining ethanol using a pipette.
- 1.2.4.8. Invert and drain the tube on clean absorbent paper. Allow the tube to air dry for 10-15 minutes.

1.2.5. DNA Hydration

- 1.2.5.1. Add 50-100 µl of 10 mM Tris-HCl, pH 8.0. Pipette up and down to dislodge the DNA pellet from the bottom and/or wall of the tube. DNA may form a diffuse pellet along the tube wall.

OPTIONAL: *An aliquot of 10 mM Tris-HCl, pH 8.0 can be warmed briefly (approx. 1-2 minutes) in a 65°C ± 1 water bath to facilitate DNA rehydration.*

- 1.2.5.2. Rehydrate DNA by incubating the sample for 1 hour at 65°C ± 1 and/or overnight at room temperature or 2-8°C. Tapping the tube periodically will aid in dispersing the DNA.

LABORATORY STANDARD OPERATING PROCEDURE FOR PULSENET TOTAL DNA EXTRACTION AND QUALITY CONTROL OF PURIFIED DNA EXTRACTS			
Doc. No. PNL33	Ver. No. 01	Effective Date:	Page 20 of 21

OPTIONAL: *Pulse spin briefly to collect sample at the bottom of the tube.*

NOTE: *Store DNA extracts at 2-8°C for short-term storage, or at -80°C to -15°C for long-term storage (>= 7 days).*

Appendix PNL33-2
Genomic DNA Clean & Concentrator-5 Kit Procedure

1. Before starting:

- 1.1. For the 25 prep kit: Add 24 ml 100% ethanol (26 ml 95% ethanol) to the 6 ml DNA Wash Buffer concentrate.
- 1.2. For the 100 prep kit: Add 96 ml 100% ethanol (104 ml 95% ethanol) to the 24 ml DNA Wash Buffer concentrate.

2. Procedure:

NOTE: All centrifugation steps should be performed between 10,000 - 16,000 x g (9,000 – 11,500 rpm).

- 2.1. In a 1.5 ml microcentrifuge tube, add 2-5 volumes of ChIP DNA Binding Buffer to each volume of DNA sample (see Table 1 below). Mix thoroughly.

Application	DNA Binding Buffer : Sample	Example
Plasmid, genomic DNA (> 2 kb)	2 : 1	200 µl : 100 µl
PCR product, DNA fragment	5 : 1	500 µl : 100 µl

Table 1: Recommended ratio of DNA Binding Buffer to Sample

- 2.2. Transfer this mixture to a provided Zymo-Spin Column¹ in a collection tube.
- 2.3. Centrifuge for 30 seconds and discard the flow-through.
- 2.4. Add 200 µl DNA Wash Buffer to the column. Centrifuge for 1 minute and repeat the wash step.
- 2.5. Add 20 µl of 10 mM Tris-HCl pH 8.0 directly to the column matrix (do not touch the filter with the pipette tip).
- 2.6. Incubate at room temperature for one minute.
- 2.7. Transfer the column to a 1.5 ml microcentrifuge tube and centrifuge for 30 seconds to elute the DNA.
- 2.8. Repeat steps 2.5 - 2.7 eluting into the same microcentrifuge to obtain a final DNA sample volume of 40 µl.
- 2.9. DNA is now ready for quantification followed by respective library preparation method.

¹The sample capacity of the column is 800 µl. It may be necessary to load and spin a column multiple times if a sample has a volume larger than 800 µl

Please proceed to respective PulseNet Library Preparation SOP.