Wet Lab Procedure for Metagenomic Sequencing Analysis

PROCEDURES

1. DNA extraction from stool samples by using DNeasy PowerSoil Pro Kit:

Equipment and Reagents to Be Supplied by User

- Microcentrifuge (up to 16,000 x g)
- Pipettor (50–1000 µl)
- TissueLyser II (cat. no. 85300) using a 2 ml Tube Holder Set (cat no. 11993)

DNeasy PowerSoil Pro Kit: Important notes before starting

- Ensure that the PowerBead Pro Tubes rotate freely in the centrifuge
- without rubbing.
- If Solution CD3 has precipitated, heat at 60°C until precipitate dissolves.
- Perform all centrifugation steps at room temperature (15–25°C).

DNeasy PowerSoil Pro Kit: Detailed Protocol

1. Spin the PowerBead Pro Tube briefly to ensure that the beads have settled at the bottom. Add up to 250 mg of soil and 800 µl of Solution CD1. Vortex briefly to mix.

Note: After the sample has been loaded into the PowerBead Pro Tube, the next step is a homogenization and lysis procedure. The PowerBead Pro Tube contains a buffer that will (a) help disperse the soil particles, (b) begin to dissolve humic acids, and (c) protect nucleic acids from degradation. Gentle vortexing mixes the components in the PowerBead Pro Tube and begins to disperse the sample in the buffer.

Homogenize samples thoroughly using one of the following methods:

Secure the PowerBead Pro Tube horizontally on a Vortex Adapter for 1.5–2 ml tubes (cat. no. 13000-V1-24). Vortex at maximum speed for 10 min.

Note: If using Vortex Adapter for more than 12 preps simultaneously, increase the vortexing time by 5–10 min.

Note: Using the Vortex Adapter will maximize homogenization, which can lead to higher DNA yields. Avoid using tape, which can become loose and result in reduced homogenization efficiency, inconsistent results, and reduced yields. (DNeasy PowerSoil Pro Kit Handbook 03/2021 13)

Use a PowerLyzer 24 Homogenizer. PowerBead Pro Tubes must be properly balanced in the tube holder of the PowerLyzer 24 Homogenizer. We recommend homogenizing the soil at 2000 rpm for 30 s, pausing for 30 s, then homogenizing again at 2000 rpm for 30 s.

Note: Homogenizing samples at higher speeds (up to 4000 rpm) may increase yields but result in more fragmented DNA.

Use a TissueLyser II. Place the PowerBead Pro Tube into the TissueLyser Adapter Set 2 x 24 (cat. no. 69982) or 2 ml Tube Holder (cat. no. 11993) and Plate Adapter Set (cat. no. 11990). Fasten the adapter into the instrument and shake for 5 min at speed 25 Hz. Reorient the

adapter so that the side that was closest to the machine body is now furthest from it. Shake again for 5 min at a speed of 25 Hz.

Note: Vortexing/shaking is critical for complete homogenization and cell lysis. Cells are lysed by a combination of chemical agents from step 1 and mechanical shaking introduced at this step. Randomly shaking the beads in the presence of disruption agents will cause the beads to collide with microbial cells and lead to the cells breaking open.

Centrifuge the PowerBead Pro Tube at 15,000 x g for 1 min.

Transfer the supernatant to a clean 2 ml Microcentrifuge Tube (provided).

Note: Expect 500–600 µl. The supernatant may still contain some soil particles.

Add 200 µl of Solution CD2 and vortex for 5 s.

Note: Solution CD2 contains IRT, which is a reagent that can precipitate non-DNA organic and inorganic material including humic substances, cell debris, and proteins. It is important to remove contaminating organic and inorganic matter that may reduce DNA purity and inhibit downstream DNA applications.

2. Centrifuge at 15,000 x g for 1 min. Avoiding the pellet, transfer up to 700 µl of supernatant to a clean 2 ml Microcentrifuge Tube (provided).

Note: Expect 500–600 µl.

Note: The pellet at this point contains non-DNA organic and inorganic material including humic acids, cell debris, and proteins. For best DNA yields and quality, avoid transferring any of the pellet.

3. Add 600 µl of Solution CD3 and vortex for 5 s.

Note: Solution CD3 is a high-concentration salt solution. Because DNA binds tightly to silica at high salt concentrations, Solution CD3 will adjust the DNA solution salt concentration to allow binding of DNA, but not non-DNA organic and inorganic material that may still be present at low levels, to the MB Spin Column filter membrane.

4. Load 650 µl of the lysate onto an MB Spin Column and centrifuge at 15,000 x g for 1 min.

Note: DNA is selectively bound to the silica membrane in the MB Spin Column in the presence of high salt solution. Contaminants pass through the filter membrane, leaving only DNA bound to the membrane.

5. Discard the flow-through and repeat step 5.3.10 to ensure that all of the lysate has passed through the MB Spin Column.

6. Carefully place the MB Spin Column into a clean 2 ml Collection Tube (provided). Avoid splashing any flow-through onto the MB Spin Column.

7. Add 500 µl of Solution EA to the MB Spin Column. Centrifuge at 15,000 x g for 1 min.

Note: Solution EA is a wash buffer that removes protein and other non-aqueous contaminants from the MB Spin Column filter membrane.

8. Discard the flow-through and place the MB Spin Column back into the same 2 ml Collection Tube.

9. Add 500 µl of Solution C5 to the MB Spin Column. Centrifuge at 15,000 x g for 1 min.

Note: Solution C5 is an ethanol-based wash solution used to further clean the DNA that is bound to the silica filter membrane in the MB Spin Column. This wash solution removes

residual salt, humic acid, and other contaminants while allowing the DNA to stay bound to the silica membrane.

10. Discard the flow-through and place the MB Spin Column into a new 2 ml Collection Tube (provided).
11. Centrifuge at up to 16,000 x g for 2 min. Carefully place the MB Spin Column into a new 1.5 ml Elution Tube (provided).

Note: This spin removes residual Solution C5. It is critical to remove all traces of Solution C5 because the ethanol in it can interfere with downstream DNA applications, such as PCR, restriction digests, and gel electrophoresis.

12. Add 50–100 µl of Solution C6 to the center of the white filter membrane.

Note: Placing Solution C6 in the center of the small white membrane will make sure the entire membrane is wet. This will result in a more efficient and complete release of the DNA from the MB Spin Column filter membrane. As Solution C6 passes through the silica membrane, DNA that was bound in the presence of high salt is selectively released by Solution C6 (10 mM Tris), which lacks salt.

13. Centrifuge at 15,000 x g for 1 min.
14. Reapply flowthrough onto spin column. Centrifuge again at 15,000 x g for 1 min.
15. Discard the MB Spin Column. The DNA is now ready for downstream applications.

Note: We recommend storing the DNA frozen (–30 to –15°C or –90 to –65°C) as Solution C6 does not contain EDTA. To concentrate DNA, refer to the Troubleshooting Guide.

DNA quantification by using Quant-iTTM 1X dsDNA HS Assay Kit

Quant-iTTM 1X dsDNA HS Assay Kit, Assay supplies needed

- Promega GloMax Discover Plate Reader
- 96-well assay plate (Corning 3651), Flat Bottom, Non binding, Black with clear bottom
- Pipettors and pipet tips

Quant-iTTM 1X dsDNA HS Assay Kit, Critical assay parameters

Assay temperature The Quant-iTTM 1X dsDNA HS Assay delivers optimal performance when all solutions are at room temperature (18–28℃). Temperature fluctuations can influence the accuracy of the assay. To minimize temperature fluctuations, insert all assay tubes into the fluorescence microplate reader only for as much time as it takes for the instrument to measure the fluorescence. Do not hold the assay tubes in your hand before reading because this warms the solution and results in a different reading.

Quant-iTTM 1X dsDNA HS Assay Kit, Incubation time

To allow the Quant-iTTM 1X dsDNA HS Assay to reach optimal fluorescence, incubate the tubes for 2 minutes after mixing the sample or the standard with the working solution. After this incubation period, the fluorescence signal is stable for 3 hours at room temperature when samples are protected from light.

Quant-iTTM 1X dsDNA HS Assay Kit, Photostability of Quant-iTTM reagents

The Quant-iTTM reagents exhibit high photostability, showing <0.3% drop in fluorescence after 9 readings and <2.5% drop in fluorescence after 40 readings.

Assay procedure IMPORTANT! For best results, ensure that all materials and reagents are at room temperature.
1. Add 10 µL of each Quant-iTTM 1X dsDNA HS Standard to separate wells. Duplicates or triplicates of the standards are recommended. We typically use duplicates in the first two columns. (ex. A1 = A2)
2. Add 1–20 µL of each unknown DNA sample to separate wells. Duplicates or triplicates of the unknown samples are recommended.
3. Load 200 µL of the Quant-iTTM 1X dsDNA working solution into each microplate well. This can be done readily using a multichannel pipettor.
4. If possible, mix your 96-well plate using a plate mixer or using the plate reader for about 3–10 seconds. Manually pipet mix up and down 20 times if no plate mixer is available. Following mixing, allow the plate to incubate at room temperature for 2 minutes.
5. Measure the fluorescence using a microplate reader (excitation/emission maxima are 502/523 nm; see Figure 1, page 2). Standard fluorescein wavelengths (excitation/emission at ~480/530 nm) are appropriate for this dye. The fluorescence signal is stable for 3 hours at room temperature when protected from light.
6. Use a standard curve to determine the DNA amounts. For the dsDNA standards, plot amount vs. fluorescence, and fit a straight line to the data points.
7. Use this workbook “QuantiFluor Analysis Tool Single Sample” https://www.promega.com/resources/tools/quantifluor-dye-systems-data-analysis-workbook/ in Excel to calculate the concentration of your samples. Copy and paste the plate results into the workbook tab “QuantiFluor Open " where you see ‘paste data below’. A standard curve should be generated on that tab. In the tab “QuantiFluor Open Results”' fill in the Dilution Factor (aqua), if applicable, and Sample Volume (green) that was loaded into each sample well. To get the correct concentration, you will need to multiply the Concentration (ng/uL) result by 10, as the spreadsheet calculation is off from Promega’s website.

Library Prep by using Illumina DNA PCR-Free Library Prep, Standard Input :
Consumables by supplier

- 10 µl pipette tips General lab supplier
- 10 µl single channel pipettes General lab supplier
- 20 µl pipette tips General lab supplier
- 20 µl multichannel pipettes General lab supplier
- 200 µl pipette tips General lab supplier
- 200 µl multichannel pipettes General lab supplier
- 200 µl single channel pipettes General lab supplier
- 1000 µl pipette tips General lab supplier
- 1000 µl single channel pipettes General lab supplier
- Hard-Shell 96-well PCR plates Bio-Rad, catalog # HSP-9601
- [Hybex] Abgene 96 well 0.8 ml polypropylene deepwell storage plates (MIDI plate) ThermoFisher Scientific, catalog # AB-0859
- 15 or 50 ml conical centrifuge tubes General lab supplier 1.5 or 1.7 ml microcentrifuge tubes General lab supplier

- Microseal 'B' adhesive seals Bio-Rad, catalog # MSB-1001
- [Optional] Microseal 'F' foil seals Bio-Rad, catalog # MSF-1001
- RNase/DNase-free multichannel reagent reservoirs, disposable VWR, catalog # 89094-658 Ethanol 200 proof (absolute) for molecular biology
- (500 ml) Sigma-Aldrich, product # E7023 Nuclease-free water General lab supplier
- [Dependent on workflow] KAPA qPCR Library Quantification Kits Roche, catalog # KK4824 or # KK4873
- Qubit dsDNA HS Assay Kit, ThermoFisher Scientific, catalog # Q32851 or # Q32854
- [Dependent on workflowl] Qubit ssDNA assay kit ThermoFisher Scientific, catalog # Q10212 Qubit Assay tubes ThermoFisher Scientific, catalog # Q32856
- [Optional] 96-well Semi-Skirted PCR plates General lab supplier

Illumina DNA PCR-Free, Standard Input : DNA Input Recommendations

The Illumina DNA PCR-Free Library Prep protocol accepts purified DNA inputs of 25–2000 ng. Follow the protocol appropriate for your input. Acceptable input types are as follows: 25-99 ng DNA—Quantification is required. Use a thermal cycler and follow the low input procedures for tagmentation and clean up. Final libraries are not normalized. Sequence low input libraries using the NovaSeq 6000 XP workflow.

100-299 ng DNA—Quantification is required. Use a thermal cycler and follow the standard input procedure for tagmentation and cleanup. The use of a Hybex incubator is not supported for ≤ 299 ng input. Final libraries are not normalized.

300-2000 ng DNA—Initial quantification is not required, but quantification of the final library pool is still required. Use a thermal cycler or Hybex and follow the standard input procedure for tagmentation and cleanup. Final libraries are normalized.

Blood/saliva—Quantification is not required. Use a thermal cycler. Final libraries are normalized. See the following section for more information on blood and saliva input requirements.

Illumina DNA PCR-Free, Standard Input : Input DNA Quantification

Quantify the starting DNA using a fluorescence-based quantification method, such as Qubit dsDNA HS. Do not use a UV spectrometer method.

Fluorescence-based methods employ a dye specific to double-stranded DNA (dsDNA) and specifically and accurately quantify dsDNA, even when many common contaminants are present. Use 2 µl for quantification

Illumina DNA PCR-Free, Standard Input : Custom Primer Considerations

The Illumina DNA PCR-Free Library Prep protocol requires the use of the VP10 primer for the Illumina DNA PCR-Free Kit, unless you are using a v1.5 or newer NovaSeq reagent kit. Follow the read 1 instructions in the NovaSeq Series Custom Primers Guide (document #1000000022266) for information on loading and setting up runs with custom sequencing primers.

Illumina DNA PCR-Free, Standard Input : Tips and Techniques

Protocol Continuity: Follow the protocol in the order described using the specified parameters. Unless a safe stopping point is specified in the protocol, proceed immediately to the next step.

Avoiding Cross-Contamination:When adding or transferring samples, change tips between each sample. When adding adapters or primers, change tips between each row and each column.

Sealing the Plate: Always seal the 96-well plate before the following steps in the protocols:

Vortexing steps

Centrifuge steps

Thermal cycling steps [Thermal Cycler Protocols]

Shaking steps [Hybex Protocol]

Apply the adhesive seal to cover the plate, and seal with a rubber roller or wedge. • Use the appropriate seal: Use Microseal 'B' adhesive seals for shaking, centrifuging, and long-term storage. The seals are effective at -40°C to 110°C and suitable for skirted or semiskirted PCR plates. Microseal 'F' adhesive foils are effective at temperatures as low as -70°C and suitable for long term storage of the 96- well plate containing final libraries. After each use, discard all seals from plates.

Plate Transfers: When transferring volumes between plates, transfer the specified volume from each well of a plate to the corresponding well of the other plate.

Centrifugation: Centrifuge at any step in the procedure to consolidate liquid or beads in the bottom of the well to prevent sample loss.

Handling Beads: When washing beads, use the DynaMag-96 Side Skirted Magnet for all PCR plates. Use of another magnet may result in suboptimal library yield. Use Magnetic Stand-96 for all MIDI plates. Dispense liquid so that no beads remain adhered to the side of the well. Keep the plate on the magnetic stand until the instructions specify to remove it. Always remove the plate from the magnetic stand before centrifuging. Do not agitate the plate while it is on the magnetic stand. Do not disturb the bead pellet. Pipette bead suspensions slowly. Immediately before use, vortex the beads until they are well dispersed. The color of the liquid must appear homogeneous. Vortex throughout protocol as necessary to keep homogenous. If beads are aspirated into pipette tips when they are not intended to be, dispense reactions back to the plate on the magnetic stand, and wait until the liquid is clear (~2 minutes).

Illumina DNA PCR-Free, Standard Input : Library Prep Workflow

Tagment Genomic DNA, Intro

This step uses the Bead-Linked Transposomes PCR-Free (BLT-PF) to tagment DNA, which is a process that fragments and tags the DNA with adapter sequences. Use the preparation steps in this section to prepare reagents in advance.

Tagment Genomic DNA, Consumables

- BLT-PF (Bead-Linked Transposomes PCR-Free)
- HP3 (2N NaOH)
- RSB (Resuspension Buffer)
- TB1 (Tagmentation Buffer 1)
- Freshly prepared 80% ethanol (EtOH)
- Nuclease-free water
- 96-well PCR plate
- Microseal 'B' adhesive seal

Tagment Genomic DNA, Prepare for a later section

- ELM (Extension Ligation Mix)
- IPB (Illumina Purification Beads)
- Index adapters (IDT for Illumina DNA/RNA UD Indexes)
- ST2 (Stop Tagment Buffer 2)
- TWB (Tagment Wash Buffer)

Tagment Genomic DNA, Warning

This set of reagents contains potentially hazardous chemicals. Personal injury can occur through inhalation, ingestion, skin contact, and eye contact. Wear protective equipment, including eye protection, gloves, and laboratory coat appropriate for risk of exposure. Handle used reagents as chemical waste and discard in accordance with applicable regional, national, and local laws and regulations.

Tagment Genomic DNA, About Reagents

Always use the specified magnetic stand to prevent failure.
BLT-PF – Store frozen and upright to make sure beads are submerged in the buffer. – Aspirate and dispense slowly due to viscosity of the solution

Tagment Genomic DNA, Preparation

Prepare the following consumables: Item Storage Instructions (* Reagent is used in a later section)

- IPB* 15°C to 30°C Vortex 1 minute. Invert 2–5 times, and then vortex thoroughly to resuspend.
- RSB 15°C to 30°C Label the tube cap RSB. Vortex or invert to mix.
- ST2 * 15°C to 30°C If precipitates are observed, heat at 37°C for 10 minutes, and then vortex until precipitates are dissolved. Vortex thoroughly, and then centrifuge.
- TWB* 15°C to 30°C Label the tube cap TWB. Invert thoroughly to mix.
- BLT-PF -25°C to -15° C Thaw at room temperature until ambient. Vortex to resuspend. Do not centrifuge before pipetting.
- ELM* -25°C to -15°C Thaw at room temperature. Keep on ice until needed. Invert to mix before use.
- HP3 -25°C to -15°C Thaw at room temperature until ambient. Vortex, and then centrifuge briefly.
- Index adapters* -25°C to -15°C Thaw at room temperature until ambient. Vortex, and then centrifuge briefly.
- TB1 -25°C to -15°C Thaw at room temperature until ambient. Vortex, and then centrifuge.

NOTE: RSB and TWB are shipped in similar tubes. Label each cap before beginning the protocol.
For each reaction, combine the following volumes to prepare diluted HP3:

- HP3 (6 µl)
- Nuclease-free water (54 µl)

These volumes produce 60 µl diluted HP3 per reaction, including overage. Vortex and centrifuge briefly.

For each reaction, combine the following volumes to prepare 80% EtOH:

- EtOH (400 µl)
- Nuclease-free water (100 µl)

These volumes produce 500 µl 80% EtOH per reaction, including overage. Vortex to mix.

Save the following TAG program on the thermal cycler:

- Choose the preheat lid option and set to 100°C
- Set the reaction volume to 50 µl
- 41°C for 5 minutes

Save the following ELM program on the thermal cycler:

- Choose the preheat lid option and set to 100°C
- Set the reaction volume to 50 µl
- 37°C for 5 minutes
- 50°C for 5 minutes

Tagment Genomic DNA, Procedure

If using gDNA input, do as follows.

1. Label a new 96-well PCR plate LP1.
2. Add 2–25 µl DNA to each well, so that the total input amount is within the desired range (100- 299 or 300-2000 ng). If sample volume is < 25 µl, bring the total volume to 25 µl using RSB.
3. Vortex BLT-PF vigorously for 1 minute to resuspend. Repeat as necessary. If beads adhere to the sides or top of the tube, briefly spin down and then pipette to resuspend.
4. [gDNA input] Add 15 µl BLT-PF to each well.
5. [Blood/Saliva input] Add 10 µl BLT-PF to each well containing 30 µl extracted DNA.
6. Using a P200 pipette set to 35 µl, pipette to mix.
7. Add 10 µl TB1 to each well.
8. Using a P200 pipette set to 40 µl, pipette to mix, and then seal.
9. Place on the preprogrammed thermal cycler and run the TAG program.
10. Proceed immediately to Post Tagmentation Cleanup.

Post Tagmentation Cleanup, Intro

This step stops the tagmentation reaction and washes the adapter-tagged DNA on the BLT-PF.

Post Tagmentation Cleanup, Consumables

- ST2 (Stop Tagment Buffer 2)
- TWB (Tagment Wash Buffer)
- Microseal 'B' adhesive seal

Post Tagmentation Cleanup, About Reagents

- Pipette TWB and ST2 slowly to minimize foaming.
- Dispense TWB directly onto beads.

Post Tagmentation Cleanup, Procedure

1. Add 10 µl ST2 to each well.
2. Using a P200 pipette set to 50 µl, pipette to mix.
3. Incubate at room temperature for 2 minutes.
4. Place on the magnetic stand and wait until the liquid is clear (~2 minutes).
5. Using a P200 pipette set to 60 µl, remove and discard all supernatant from each well.
6. Remove the plate from the magnetic stand.
7. Add 150 µl TWB to each well.
8. Pipette to mix ten times or until beads are fully resuspended.
9. Place on the magnetic stand and wait until the liquid is clear (~2 minutes).
10. Optional: Check tagmented DNA by running the samples on the Agilent Bioanalyzer 2100 using Agilent DNA 7500 kit. See attached Agilent protocol.

Illumina DNA PCR-Free, Ligate Indexes, Intro

This step ligates Index 1 (i7) and Index 2 (i5) adapters to each sample.

Ligate Indexes, Consumables

- ELM (Extension Ligation Mix)
- Index adapters (IDT for Illumina DNA/RNA UD Indexes)
- TWB (Tagment Wash Buffer)
- Diluted HP3
- Microseal 'B' adhesive seals

Ligate Indexes, About Reagents

- The index plate wells cannot be reused.
- Aspirate and dispense ELM slowly due to the viscosity of the solution.

Ligate Indexes, Procedure

1. Without disturbing the bead pellet, remove and discard residual supernatant from each well.
2. Remove the plate from the magnetic stand.
3. Add 45 µl ELM to each well.
4. Pipette to mix until beads are fully resuspended and not on the side of the well.
5. Pierce the foil seal covering the index adapter plate as follows.
   [< 96 samples] Pierce the wells you intend to use. Use a new pipette tip for each well.
   [96 samples] Align a new semi-skirted 96-well PCR plate over the index adapter plate and slowly press down to puncture all 96 wells. Discard the PCR plate.
6. Add 5 µl index adapters to each well.
7. Using a P200 pipette set to 45 µl, pipette to mix, and then seal.
8. Place on the preprogrammed thermal cycler and run the ELM program.

9. Place on the magnetic stand and wait until the liquid is clear (~2 minutes).
10. Remove and discard all supernatant from each well.
11. Remove the plate from the magnetic stand.
12. Add 75 µl TWB onto the beads in each well.
13. Pipette to mix until beads are fully resuspended and not on the side of the well.
14. Place on the magnetic stand and wait until the liquid is clear (~2 minutes).
15. Remove and discard all supernatant from each well.
16. Seal, and then centrifuge at 280 x g for 10 seconds.
17. Place on the magnetic stand.
18. Without disturbing the bead pellet, remove and discard residual supernatant from each well.
19. Remove the plate from the magnetic stand.
20. Add 45 µl diluted HP3 to each well.
21. Pipette to mix until beads are fully resuspended and not on the side of the well.
22. Incubate at room temperature for 2 minutes.
23. Proceed immediately to Clean Up Libraries.

Illumina DNA PCR-Free, Clean Up Libraries, Intro

This step uses a double-sided bead purification procedure to purify the libraries. In the first side of the bead purification procedure, Illumina purification beads are added to the sample containing BLT-PF beads. Then the supernatant is transferred to a new plate containing Illumina purification beads for the second side of the double-sided bead purification procedure.

Clean Up Libraries, Consumables

- IPB (Illumina Purification Beads)
- RSB (Resuspension Buffer)
- Freshly prepared 80% ethanol (EtOH)
- 96-well PCR plate (2)
- Microseal 'B' adhesive seal
- [Optional] Microseal 'F' adhesive foil

Clean Up Libraries, About Reagents

- IPB: Vortex before each use. Vortex frequently to make sure that beads are evenly distributed. Aspirate and dispense slowly due to the viscosity of the solution.

Clean Up Libraries, Procedure

1. Vortex IPB.
2. Add 36 µl IPB to each well containing sample with BLT-PF beads.
3. Pipette 10 times to mix.
4. Incubate at room temperature for 2 minutes.
5. Place on the magnetic stand and wait until the liquid is clear (~5 minutes).
6. While the plate incubates, label a new 96-well PCR plate LP2.
7. Vortex and then invert IPB until fully resuspended.

8. Add 42 µl IPB to each well of LP2.
9. Without disturbing the bead pellet, transfer 76 µl supernatant from each well of LP1 to the corresponding well of the LP2.
10. Pipette to mix until all beads are in solution.
11. Remove LP1 from the magnetic stand, and then discard.
12. Incubate LP2 at room temperature for 2 minutes.
13. Place on the magnetic stand and wait until the liquid is clear (~5 minutes).
14. Without disturbing the bead pellet, remove and discard all supernatant from each well.
15. Wash beads as follows.
    - Keep on the magnetic stand and add 180 µl fresh 80% ethanol to each well.
    - Wait 30 seconds.
    - Remove and discard all supernatant from each well.
16. Wash beads a second time.
17. Apply Microseal 'B' and then centrifuge 280 x g for 10 seconds.
18. Place on the magnetic stand, and then wait 10 seconds.
19. Remove residual EtOH from each well.
20. Discard unused 80% EtOH.
21. Air-dry on the magnetic stand (~2 minutes).
22. Remove from the magnetic stand.
23. Add 22 µl RSB onto the beads in each well.
24. Pipette to mix until beads are fully resuspended and not on the side of the well.
25. Incubate at room temperature for 2 minutes.
26. Centrifuge 280 x g for 10 seconds.
27. Place on the magnetic stand and wait until the liquid is clear (~2 minutes).
28. Label a new PCR plate FLP.
29. Transfer 20 µl supernatant from each well of LP2 to the corresponding well of FLP.
30. Proceed to Quantify and Pool Libraries.

SAFE STOPPING POINT

If you are stopping, seal the plate with Microseal 'B' or Microseal 'F' and store at -25°C to -15°C for up to 30 days.

Library quantification by using Illumina DNA PCR-Free, Quantify and Pool Libraries

Pool equal library volumes and quantify the pool before sequencing to ensure optimal cluster density (for gDNA inputs >300 ng and whole blood, dried blood spots, and saliva sample input protocols only). Illumina DNA PCR-Free Library Prep libraries are single stranded. It is not possible to view library size distribution on any capillary electrophoresis instrument.
For pooling guidelines, see Index Adapter Pooling Guide (document # 1000000041074).

≥ 300ng DNA Input

For DNA inputs ≥ 300 ng, quantifying and normalizing libraries from the same experiment is not required. If using libraries from separate experiments, the final yields can vary. For each sequencing library pool, combine 9µl of each library in a 1.5 or 1.7 ml microcentrifuge tube. Vortex to mix, and then centrifuge at 280 x gfor 1 minute.

Quantify the library pool: Analyze 2 µl pooled library using the Qubit ssDNA (single- stranded) assay kit or a KAPA qPCR Library Quantification Kit.

Dilute Libraries to the Starting Concentration and Sequence, Intro

This step dilutes libraries to the starting concentration for your sequencing system and is the first step in a serial dilution. After diluting to the starting concentration, libraries are ready to be denatured and diluted to the final loading concentration.

Dilute Libraries to the Starting Concentration and Sequence, Procedure

1. Calculate the volumes of RSB and library pool (for inputs ≥ 300 ng) or single libraries (for inputs 100- 299 ng) to dilute libraries to the starting concentration for your system.
2. When using a qPCR method—Use the molarity value determined by the KAPA qPCR protocol. Use 450 bp as the average library size and 660 g/mol as the DNA mass. Illumina recommends diluting libraries by a 1:10000x dilution when using the KAPA method.
3. When using Qubit method — Calculate the molarity value of the pooled libraries using the formula below.
    a. The formula uses 450 bp as the average library size and 660 g/mol as the DNA mass. This equation will output the double stranded DNA equivalent.
    Molarity(nM) =Yield(ng/uL) x 3.36
4. Dilute the pool to the starting concentration for your system.
5. If you are using a v1.0 NovaSeq reagent kit, prepare the VP10 custom sequencing primer. See Custom Primer Considerations for more information on using custom sequencing primers with Illumina DNA PCR-Free. Follow the standard workflow instructions in Protocol A or the XP workflow instructions in Protocol B of the the NovaSeq Denature and Dilute Libraries Guide (document # 1000000106351) to dilute to the final loading concentration.

For sequencing, Illumina recommends setting up a paired-end run of 151 cycles per read using 10 bp index reads: (Read 1, i5 index read, i7 index read, Read 2), (151, 10, 10, 151). See the NovaSeq 6000 Sequencing System Guide (document # 1000000019358) for additional sequencing information.

Quantification of libraries by using Qubit® ssDNA Assay Kit

Qubit® ssDNA Assay Kit: Before you Begin

Materials required but not provided:

- Plastic container (disposable) for mixing the Qubit® working solution (step 1.3, page 3).
- Qubit® assay tubes (500 tubes, Life Technologies, Cat. no. Q32856) or Axygen® PCR-05-C tubes (VWR, part no. 10011-830)

Storing the Qubit® assay kits

The Qubit® ssDNA Reagent and Buffer are designed for room temperature storage. The Qubit® ssDNA Reagent is supplied in DMSO, which freezes at temperatures lower than room temperature. Store the ssDNA standards at 4°C.

Critical assay parameters

Assay temperature

- The Qubit® ssDNA Assay delivers optimal performance when all solutions are at room temperature (22–28˚C).
- The Qubit® assays are designed to be performed at room temperature, as temperature fluctuations can influence the accuracy of the assay. To minimize temperature fluctuations, store the Qubit® ssDNA Reagent and Buffer at room temperature and insert all assay tubes into the Qubit® Fluorometer only for as much time as it takes for the instrument to measure the fluorescence; the Qubit® Fluorometer can raise the temperature of the assay solution significantly, even over a period of a few minutes.
- Do not hold the assay tubes in your hand before reading because this warms the solution and results in a low reading.

Incubation time

- To allow the Qubit® assay to reach optimal fluorescence, incubate the tubes for the DNA assays for 2 minutes after mixing the sample or standard with the working solution. After this incubation period, the fluorescence signal is stable for 30 minutes at room temperature.

Photobleaching of the Qubit® reagents

- The Qubit® reagents exhibit high photostability in the Qubit® Fluorometer, showing <0.3% drop in fluorescence after 9 readings and <2.5% drop in fluorescence after 40 readings. However, if the assay tube remains in the Qubit® Fluorometer for multiple readings, a temporary reduction in fluorescence will be observed as the solution increases in temperature. Note that 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.

Calibrating the Qubit® Fluorometer

- For each assay, you have the choice to run a new calibration or use the values from the previous calibration. When you first use the instrument, perform a new calibration each time. As you become familiar with the assays, the instrument, your pipetting accuracy, and significant temperature fluctuations within your laboratory, you can decide how comfortable you are using the calibration data stored from the last time the instrument was calibrated. Additionally, remember that the fluorescence signal in the tubes containing standards and samples is stable for no longer than 30 minutes. See Figure 1 (page 6) for an example of the calibration curve used to generate the quantification results.

Handling and disposal

- No data are currently available that address the mutagenicity or toxicity of the Qubit® ssDNA Reagent (Component A). This reagent is known to bind nucleic acid and is provided as a solution in DMSO. Treat the Qubit® ssDNA Reagent with the same safety precautions as all other potential mutagens and dispose of the dye in accordance with local regulations.

Qubit® ssDNA Assay Kit: Preparing samples and standards

This protocol assumes that you are preparing standards for calibrating the Qubit® Fluorometer. If you plan to use the last calibration performed on the instrument (see “Calibrating the Qubit® Fluorometer” on page 2), you need fewer tubes (step 1.1) and less working solution (step 1.3).

1. Set up the required number of 0.5-mL tubes for standards and samples. The Qubit® ssDNA Assay requires 2 standards. Note: Use only thin-wall, clear, 0.5-mL PCR tubes. Acceptable tubes include Qubit® assay tubes (Cat. no. Q32856) or Axygen® PCR-05-C tubes (part no. 10011-830).
2. Label the tube lids Note: Do not label the side of the tube as this could interfere with the sample read. Label the lid of each standard tube correctly. Calibration of the Qubit® Fluorometer requires the standards to be inserted into the instrument in the right order.
3. Prepare the Qubit® working solution by diluting the Qubit® ssDNA Reagent 1:200 in Qubit® ssDNA Buffer. Use a clean plastic tube each time you prepare Qubit® working solution. Do not mix the working solution in a glass container.
    a. Note: The final volume in each tube must be 200 µL. Each standard tube requires 190 µL of Qubit® working solution, and each sample tube requires anywhere from 180–199 µL. Prepare sufficient Qubit® working solution to accommodate all standards and samples. For example, for 8 samples, prepare enough working solution for the samples and 2 standards: ~200 µL per tube in 10 tubes yields 2 mL of working solution (10 µL of Qubit® reagent plus 1990 µL of Qubit® Buffer).
4. Add 190 µL of Qubit® working solution to each of the tubes used for standards.
5. Add 10 µL of each Qubit® standard to the appropriate tube, then mix by vortexing 2–3 seconds. Be careful not to create bubbles.
    a. Note: Careful pipetting is critical to ensure that exactly 10 µL of each Qubit® standard is added to 190 µL of Qubit® working solution.
6. Add Qubit® working solution to individual assay tubes so that the final volume in each tube after adding sample is 200 µL.
    a. Note: Your sample can be anywhere from 1–20 µL. Add a corresponding volume of Qubit® working solution to each assay tube: anywhere from 180–199 µL.
7. Add each sample to the assay tubes containing the correct volume of Qubit® working solution, then mix by vortexing 2–3 seconds. The final volume in each tube should be 200 µL.
8. Allow all tubes to incubate at room temperature for 2 minutes.
9. Proceed to “Reading standards and samples”; follow the procedure appropriate for your instrument.
10.

Qubit® ssDNA Assay Kit: Reading standards and samples (Qubit® 3.0 Fluorometer)

1. On the Home screen of the Qubit® 3.0 Fluorometer, press DNA, then select ssDNA as the assay type. The “Read standards” screen is displayed. Press Read Standards to proceed.

    a. Note: If you have already performed a calibration for the selected assay, the instrument prompts you to choose between reading new standards and running samples using the previous calibration.
2. Insert the tube containing Standard #1 into the sample chamber, close the lid, then press ‘Read standard’. When the reading is complete (~3 seconds), remove Standard #1.
3. Insert the tube containing Standard #2 into the sample chamber, close the lid, then press ‘Read standard’. When the reading is complete, remove Standard #2.
4. The instrument displays the results on the Read standard screen. For information on interpreting the calibration results, refer to the Qubit® 3.0 Fluorometer User Guide.
5. Press ‘Run samples’.
6. On the assay screen, select the sample volume and units:
7. Press the + or – buttons on the wheel to select the sample volume added to the assay tube (from 1–20 µL).
8. From the dropdown menu, select the units for the output sample concentration.
9. Insert a sample tube into the sample chamber, close the lid, then press ‘Read tube’. When the reading is complete (~3 seconds), remove the sample tube.
10. The instrument displays the results on the assay screen. The top value (in large font) is the concentration of the original sample. The bottom value is the dilution concentration. For information on interpreting the sample results, refer to the Qubit® 3.0 Fluorometer User Guide.
11. Repeat step 5.11.3.8 until all samples have been read.

Preparing Illumina NextSeq 2000 Reagent Cartridge: Thaw the Bagged Cartridge and Flow Cell

This step thaws the cartridge in the unopened bag and prepares the flow cell. Thaw the bagged cartridge using one of three methods: controlled water bath, refrigerator, or room temperature air. Use the cartridge immediately after thawing, without refreezing. If you are unable to use the cartridge immediately after thawing, see Return Consumables to Storage.

Thaw Cartridge in a Controlled Water Bath

1. Put on a new pair of powder-free gloves and remove the cartridge from storage.
2. Remove the cartridge from the box but do not open the silver foil bag. Thawing a torn or punctured bag in a water bath can result in sequencing failure. Thaw at room temperature or in a refrigerator instead.
3. Thaw the bagged cartridge in a controlled 25°C water bath for 6 hours:
4. Maintain a water depth of at least 9.5–10 cm regardless of how many cartridges you are thawing.

5. Set a temperature-controlled water bath to 25°C.
6. Face the bag label up and set in a water bath without submerging.
7. Do not attempt to weigh the cartridge down to submerge. If the bag label is not facing up or the cartridge inverts during thawing, sequencing data will be negatively impacted.
8. Do not exceed 8 hours in the water bath.
9. Do not simultaneously thaw more cartridges than supported by the water bath.

10. Do not stack cartridges.
11. Remove cartridge from water bath and dry with paper towels.

Thaw Cartridge in a Refrigerator

1. Put on a new pair of powder-free gloves.
2. One day prior to anticipated run, remove the cartridge from -25°C to -15°C storage.
3. Remove the cartridge from the box but do not open the silver foil bag.
4. Position the cartridge at room temperature so that the label faces up and air can circulate on sides and top. If the bag label is not facing up, sequencing data will be negatively impacted.
5. Thaw at room temperature for 6 hours.
6. Position the cartridge in a 2°C to 8°C refrigerator so that the label faces up and air can circulate on sides. If the bag label is not facing up, sequencing data will be negatively impacted.
7. Thaw in the refrigerator for 12 hours. Do not exceed 72 hours.

Thaw Cartridge at Room Temperature

1. Put on a new pair of powder-free gloves.
2. Remove the cartridge from -25°C to -15°C storage.
3. Remove the cartridge from the box but do not open the silver foil bag.
4. Position the cartridge so that the label faces up and air can circulate on sides and top. If the bag label is not facing up, sequencing data will be negatively impacted.
5. Thaw at room temperature for 9 hours. Do not exceed 16 hours.
6. Prepare the Flow Cell and Cartridge
7. Prepare the flow cell as follows.
8. Remove a new flow cell from 2°C to 8°C storage.
9. Set the unopened package aside at room temperature for 10–15 minutes to prevent condensation when removing the flow cell from the package. Preparing the flow cell now ensures that it reaches room temperature on time.

If using the refrigerator thaw method:

1. Remove thawed cartridge from 2°C to 8°C storage.
2. Set the unopened cartridge aside at room temperature for at least 15 minutes before sequencing. Do not exceed 1 hour.

Illumina NextSeq 2000 Denature and Dilute Libraries

Loading Volume and Concentration

- This procedure denatures and dilutes libraries to a final loading volume of 200 µl. Loading concentration can vary depending on library preparation and quantification methods.

Consumables: the following consumables are required to denature and dilute libraries

- Disposable gloves, powder free
- PhiX Control v3 Illumina (Cat. No. FC-110-3001)

- Illumina® DNA PCR-Free Prep Sequencing Primers Read 1 + Index 2 (Cat. No. 20041797)
- Microcentrifuge tube, 1.5 ml, Low bind

The following consumables for the denaturing and diluting libraries and PhiX are provided in the NextSeq 1000/2000 Reagents kits and Illumina library prep kits.

- NextSeq 1000/2000 RSB with Tween 20 Illumina provided in the NextSeq 1000/2000 reagents kits, with exception to the NextSeq 1000/2000 P2 (v2) Reagents kit.

Equipment

- Centrifuge General lab supplier
- Vortexer General lab supplier

Dilute Libraries

If using onboard denature and dilute, this step dilutes libraries to the applicable loading concentration. An optional 2% PhiX1spike-in provides additional metrics, base diversity, or a positive control. The PhiX spike-in percentage should be increased for libraries with lower base diversity. If manually denaturing and diluting libraries, use the NextSeq 1000 and 2000 Denature and Dilute Libraries Guide (document # 1000000139235). This step only applies to onboard denature and dilution.

1. Dilute Library to 2 nM
2. Dilute 2 nM Library to Loading Concentration (900pM)
3. Calculate volumes in a low-bind microtube to prepare a 24µl library diluted to the appropriate loading concentration (900pM).
4. Vortex briefly, and then centrifuge at 280 × g for 1 minute. Set aside the diluted library on ice until ready for sequencing. Sequence libraries diluted to the loading concentration the same day they are diluted.
5. Remove 10 nM PhiX stock from -25°C to -15°C storage. PhiX is needed only for an optional spike-in or a PhiX-only run, which is done in this protocol.
6. [Optional] Thaw PhiX at room temperature for 5 minutes, and then quantify using a fluorescence based method, such as Qubit, to confirm PhiX concentration. If quantification is not possible, proceed with 10 nM concentration.
7. Vortex library or PhiX briefly, and then centrifuge at 280 × g for 1 minute.
8. Using RSB with Tween 20 as diluent, prepare at least 24 µl 2 nM library in a low-bind microtube.
9. Combine the following volumes in a low-bind microtube to prepare 20 µl 1 nM PhiX:
    a. 10 nM PhiX (2 µl)
    b. RSB with Tween 20 (18 µl)
10. Vortex briefly, and then centrifuge at 280 × g for 1 minute.
11. Add 1 µl 1 nM PhiX to 24 µl library diluted to the final loading concentration. These volumes result in a ~2% PhiX spike-in. Actual percentage varies depending on library quality and quantity.
12. Set aside the library with PhiX spike-in on ice until ready for sequencing.
13. Sequence libraries with PhiX spike-in the same day they are diluted.

Load Consumables into the Cartridge

1. Cartridge: Open the cartridge bag by tearing or cutting with scissors from the top notch on either side.
2. Remove the cartridge from the bag. Discard the bag and desiccant.
3. Invert the cartridge 10 times to mix reagents. Internal components can rattle during inversion, which is normal.
4. Flow Cell: Open the silver foil package by tearing or cutting with scissors from the top slit on either side. If unable to use the flow cell immediately, see Return Consumables to Storage.
5. Pull the flow cell out of the package. Set aside the foil package and desiccant in case you need to return the flow cell to storage. The desiccant is contained in a pouch at the bottom of the foil package. Discard them when sequencing begins.
6. Hold the flow cell by the gray tab with the label on the tab facing up. Push to insert the flow cell into the slot on the front of the cartridge. An audible click indicates that the flow cell is in place. When properly loaded, the gray tab protrudes from the cartridge.
7. Pull back and remove the gray tab to expose the flow cell. Recycle the tab.

Loading: Load Libraries

1. Using a new P1000 pipette tip, pierce the Library reservoir and push the foil to the edges to enlarge the hole.
2. Discard the pipette tip to prevent contamination.
3. Add 20 µl diluted library to the bottom of the reservoir by slowly lowering the pipette tip to the bottom of the reservoir before dispensing. Avoid touching the foil.
4. Load Illumina® DNA PCR-Free Prep Sequencing Primers Read 1 + Index 2
    a. Add 600uL Read 1 Primer (VP10) to the #1 well in the cartridge.
    b. Add 600uL Index 2 Primer (VP14) to the #2 well in the cartridge.

Illumina NextSeq 2000 Start Run

1. Create Sample Sheet using Illumina BaseSpace Sequence Hub
2. Create an account or Log in (basespace.illumina.com)
3. Runs>New Runs>Instrument Run Setup
4. Fill in all information required, including sample information, and Download and save as <run name>.csv file.
5. Upload .csv Sample Sheet to server environment.
    a. /identify/path/here
6. Initiate Sequencing Run in Local Run mode
7. Select Start.
8. Select Choose... under Start With Sample Sheet, and navigate to the sample sheet in v2 formatting on the NextSeq 1000/2000 instrument, portable drive, or mounted network drive. Sample sheet file names cannot contain special characters.
9. NextSeq 1000/2000 Control Software v1.3 or later automatically detects the DRAGEN version from the sample sheet and prompts you to switch versions, if needed. The DRAGEN version must be installed on the system. For installation information, see Software Updates.

10. The selected sample sheet must be in v2 formatting. To create a sample sheet v2, download the generated sample sheet from Instrument Run Setup in BaseSpace Sequence Hub or edit a sample sheet v2 template provided on the NextSeq 1000/2000 support page. See Sample Sheet v2 Settings for more information on sample sheet v2 formatting and requirements. Make sure any files referenced in the sample sheet are located in the same folder as the sample sheet.
11. Select Review.
12. Enter custom read primer and custom index primer locations. Choose
    a. "Read 1 Primer Yes" and
    b. "Index 2 Primer Yes"
13. Choose "0" for Barcode Mismatch in run setup
14. For information on preparing and adding custom primers, see the NextSeq 1000 and 2000 Custom Primers Guide (document # 1000000139569). Make sure to visit the Compatible Products page for your library prep kit to check if Illumina custom primers are necessary.
15. [Optional] Select a custom recipe. For more information, see Dark Cycle Sequencing on page 99 If using the NextSeq 1000/2000 Control Software v1.3 or later and the Illumina Stranded Total RNA Prep with Ribo-Zero Plus kit or the Illumina Stranded mRNA Prep kit, the custom recipe is automatically selected.

16. [Optional] To manually denature and dilute libraries, deselect the Denature and Dilute On Board checkbox. See the NextSeq 1000 and 2000 Denature and Dilute Libraries Guide (document # 1000000139235). The default selection is configured in the NextSeq 1000/2000 Control Software settings.
17. [Optional] To change the output folder, select the Output Folder field and enter a new location. The Output Folder field is auto-populated from your default settings and is required unless Proactive, Run Monitoring and Storage is selected.

Load the Consumables Onto the Instrument

1. Make sure that the cartridge was previously thawed and inverted 10 times to mix prior to loading the flow cell (gray tab removed) and diluted library.
2. Select Load. The NextSeq 1000/2000 Control Software opens the visor and ejects the tray.
3. Place the cartridge onto the tray with the label facing up and the flow cell inside the instrument. Push the cartridge in until it locks into the seat. Select Close to retract the cartridge and close the visor. The NextSeq 1000/2000 Control Software displays information from the scanned consumables after ~3 minutes.
4. [Optional] Select Eject Cartridge to remove the cartridge. The visor opens after 1 minute and ejects the cartridge.
5. Select Sequence.
6. Pre-Run Checks. Pre-run checks include an instrument check followed by a fluidics check. The fluidics check pierces the cartridge seals, which will cause 3-4 popping sounds to emit from the instrument. This is expected. The reagent is now passed through the flow cell.

7. Consumables cannot be reused once the fluidics check starts.
8. Wait about 15 minutes for pre-run checks to complete.
9. The run starts automatically after successful completion.
    a. If an error occurs during the instrument check, select Retry to redo the check. When a check is in progress, the circle for that check is animated.
    b. To troubleshoot recurring errors, see Error Message Resolution.

For additional instructions for operating the NextSeq2000, refer to the *NextSeq 1000/2000 Product Documentation* found at NextSeq 1000/2000 Product Documentation