Protocol for Spiking in Standards to DNA Extracts

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Citation

Langenfeld, Kathryn, et al. "A quantitative metagenomic approach to determine population concentrations with examination of quantitative limitations." *bioRxiv* (2022): 2022-07.

Materials

- DNA Extracts for Sequencing
- Synthetic dsDNA standard mix (a sequins metagenome mix from sequinstandards.com/metagenome/)
- *(If sequencing ssDNA)* Synthetic ssDNA standards
- Qubit dsDNA HS assay (or another method to measure dsDNA concentrations)
- *(If sequencing ssDNA)* Qubit ssDNA assay (or another method to measure ssDNA concentrations)

Steps

1. Resuspend dsDNA and, if applicable, ssDNA standards. Aliquot standards and store at -20˚C to limit the number of freeze thaw cycles that standards go through.
2. Thaw standards and DNA extracts on ice. Keep standards and DNA extracts on ice between steps.
3. *(If using ssDNA standards)* Measure the ssDNA concentrations of each ssDNA standard. Create a mix of ssDNA standards that vary in concentration. Create a ssDNA standard mix volume that is approximately required for spiking into the DNA extracts. Dilute the individual standards as needed to create the mix volume. Recommend selecting dilutions such that only the upper half of volumes for pipettes are used to increase the accuracy of pipetted volumes.

Table 1 Concentrations of ssDNA standards added to the mix used in Langenfeld et al. (2023).

ssDNA Standard	Mix Concentration (copies/µL)
NC_000936.1_S3	1.00E+07
NC_027637.1_S2	1.00E+04
NC_039057.1_S1	1.00E+06
NC_025708.1_S1	1.00E+08
NC_010429.1_S4	1.00E+05

4. Immediately prior to spiking in standards, measure the dsDNA concentrations in the DNA extracts and dsDNA standards mix. Langenfeld et al. (2023) used Qubit dsDNA HS Assay to quantify dsDNA concentrations.
5. *(If sequencing ssDNA)* Immediately prior to spiking in standards, measure the ssDNA concentrations in the DNA extracts and the ssDNA standards mix. Langenfeld et al. (2023) used Qubit ssDNA Assay. The Qubit ssDNA Assay measures total nucleic acids. Therefore, the true ssDNA concentration is the difference between the ssDNA measurement and the dsDNA concentration.

6. Spike in dsDNA standards into the sample. Dilute standards as necessary so spike-in volumes are in the upper half of pipette volumes. Langenfeld et al. (2023) spiked in the dsDNA standard mix such that the lowest standard concentration was 10 copies/ng DNA extract. We suggest changing this spike-in concentration depending on the sequencing depth and anticipated microbiome complexity.

Table 2 Calculate the spike-in volumes of the dsDNA standards mix. The last column is the Pre-spike DNA Concentration * dsDNA Standards Spike-in Concentration * Dilution / dsDNA Standards Mix Concentration.

Sample	Pre-spike DNA Concentration (ng/µL)	dsDNA Standards Mix				
		Mix Concentration (ng/µL)	Mix Concentration (lowest standard copies/µL)	Spike-in Concentration (lowest standard copies/ng)	Dilution	Spike-in Volume (µL std stock/µL sample)
Example	41.4	2.19	2.14E+03	10	1	1.93E-01

7. *(If sequencing ssDNA)* Spike in ssDNA standards into the sample. Dilute standards as necessary so spike-in volumes are in the upper half of pipette volumes. Langenfeld et al. (2023) spiked in the ssDNA standard mix such that the lowest standard concentration was 10 copies/ng DNA extract. We suggest changing this as necessary depending on the sequencing depth and anticipated microbiome complexity.

Table 3 Calculate the spike-in volumes of the ssDNA standards mix. The last column is the Pre-spike DNA Concentration * ssDNA Standards Spike-in Concentration * Dilution / ssDNA Standards Mix Concentration.

Sample	Pre-spike DNA Concentration (ng/µL)	ssDNA Standards Mix				
		Mix Concentration (ng/µL)	Mix Concentration (lowest standard copies/µL)	Spike-in Concentration (lowest standard copies/ng)	Dilution	Spike-in Volume (µL std stock/µL sample)
Example	41.4	5.70E-02	1.00E+04	10	5	2.07E-01