nature protocols

https://doi.org/10.1038/s41596-025-01165-5

Protocol

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Absolute quantification of prokaryotes in the microbiome by 16S rRNA qPCR or ddPCR

In the format provided by the authors and unedited

Supplementary Information

Supplementary Methods

Supplementary Methods 1 – Choosing an amount of dilution

The purpose of Supplementary Methods 1 is to provide guidance on selecting the amount of sample DNA dilution needed for either qPCR or ddPCR. The principles incorporated in these suggestions should be broadly applicable, but the specific dilution amounts given may need to be modified. For this reason, it may be helpful to first assay a representative subset of samples.

Dilution for qPCR

The ideal range of 16S rRNA copies per reaction in qPCR is approximately 5×10^3 to 10^8 given that the limit of blank is approximately 5×10^1 to 5×10^2. We recommend first assaying all samples at a $1{:}10^3$ dilution.

If a sample is too concentrated according to qPCR-specific analysis, it should be re-assayed with an additional ten-fold dilution.

If a sample is too dilute according to qPCR-specific analysis, it should be re-assayed with less dilution.

- The most comprehensive approach is to measure the sample without dilution, with a 1:10 dilution, and with a 1:100 dilution. This maximizes the likelihood of the sample falling within the quantification range and, if multiple dilutions fall within the quantification range, this will provide information on any potential PCR inhibition present in the sample.
- Generally, the likelihood of PCR inhibition decreases as sample dilution increases. For this reason, we recommend choosing the most dilute measurement that falls within the quantification range and is not labeled low confidence by qPCR-specific analysis.

For more details on the analysis, refer to qPCR-specific analysis (Steps 49Axxiii-49Axxxix) and Shared analysis and quality control (Steps 50-65) in the main protocol.

Dilution for ddPCR

The quantification range in ddPCR is narrower and lower, with values ideally falling between approximately 10^2 and 10^5 16S rRNA copies per reaction given that the limit of blank is 10^0 to 2.5×10^1. As such, the optimal dilution strategy will vary depending on the expected concentration of your samples. For high abundance stool samples (e.g. healthy donors), we recommend first assaying the extracted DNA with a $1{:}10^5$ dilution. For stool samples with lower biomass (e.g. some clinical cohorts), we recommend first assaying the extracted DNA with a $1{:}10^4$ dilution.

If a sample is too concentrated according to ddPCR-specific analysis, it should be re-assayed with an additional ten-fold dilution.

If a sample is too dilute according to ddPCR-specific analysis, it should be re-assayed with less dilution.

- For the same reasons as in the qPCR, we recommend measuring the sample without dilution, with a 1:10 dilution, and with a $1:10^2$ dilution.

For more details on the analysis, refer to ddPCR-specific analysis (Steps 49Bxiv-49Bxviii) and Shared analysis and quality control (Steps 50-65) in the main protocol.

NOTE: It is straightforward to assay a given set of samples at multiple dilutions or to assay some samples at one dilution and others at another. To do this, transfer samples from the intermediate liquid handler dilution plates and combine onto a single sample plate for qPCR technical replicate generation or ddPCR plate generation.

Supplementary Methods 2 – Alternative dilution process

In the absence of a liquid handler, serial dilutions may be performed in the 96-well format utilizing a liquidator (e.g. Mettler Toledo Liquidator 96) or using a multichannel pipettor. With a liquidator, deep 96-well plates may be used allowing for larger total volumes to reduce the number of dilution steps. With a multichannel pipettor, we recommend using one pipettor to transfer the small, concentrated volume and another pipettor set to approximately half the total volume for mixing dilution points. In all cases, it is essential that the correct volume is transferred and even more critical that thorough mixing occurs.

In Dilute sample DNA in 96-well format (Steps 35-48) in the main protocol, we describe our process for using a liquid handler to serially dilute samples in steps of 1:10 with a total volume at each step of 50 µL. This process has the advantages of (1) transferring a relatively large volume, (2) being able to mix almost half of the total volume with 20 µL liquid handler pipette tips, and 3) being adaptable to different amounts of dilution.

As an alternative, we have also used the following process to dilute 1:50 and then 1:20 for a total of $1:10^3$. The advantage of this strategy is that less extracted DNA volume is needed and fewer plates are used. Here are the details for this alternative program:

NOTE: Perform Steps 1-3 of Supplementary Methods 2 in the PCR workstation.

1. Follow Steps 35-38 of the main protocol.
2. Label a 96-well plate “undiluted sample”. Aliquot 4 µL of samples, extraction controls, NTCs, and NIST positive PCR controls (pre-diluted per Steps 37 and 38) to the undiluted sample plate wells corresponding to the plate layout from Step 35. Do not aliquot the standard curve dilution points, if applicable, at this time. Use a full box of pipette tips in the corresponding order to reduce the risk of error. Seal the undiluted sample plate with an adhesive foil seal.
3. Label two 96-well plates “1:50” and “1:20”. Pour nuclease-free water into a sterile reservoir. Aliquot 98 µL of nuclease-free water from the reservoir to each well of the 1:50 plate and 95 µL of nuclease-free water from the reservoir to each well of the 1:20 plate using a multichannel pipettor. Avoid pipetting water into the well locations corresponding to the standard curve dilution points, if applicable. Seal each plate with an adhesive foil seal.

4. Transport the three sealed plates to the liquid handler with two boxes of liquid handler pipette tips (one box per serial dilution transfer) and one adhesive foil seal.
5. Follow Steps 42-44 of the main protocol.
6. The liquid handler puts on pipette tips and transfers 2 µL from the undiluted sample plate to the 1:50 plate.
7. Follow Step 46 of the main protocol.
8. The pipette tip box is then replaced, and the liquid handler transfers 5 µL from the 1:50 plate to the 1:20 plate.
9. Follow Step 46 of the main protocol.
10. Seal the 1:20 plate with an adhesive foil seal and keep on ice until needed. This plate will be referred to as the "final sample dilution plate" hereafter.
11. Resume the main protocol from Step 49.

Supplementary Methods 3 – Dilution process testing

Supplementary Methods 3 focuses on testing the prepared dilution process, regardless of the equipment. For simplicity within Supplementary Methods 3, the first dilution plate is referred to as the 1:10 plate and the second dilution plate is referred to as the 1:100 plate, but this testing process can be applied even if the dilution amounts are different.

Water-based testing of program parameters

We recommend checking that your dilution process is set up properly.

1. Pour Milli-Q water into a reservoir. With a multichannel pipettor, aliquot the same volume of Milli-Q as you would sample onto the undiluted sample plate.
2. Fill two additional 96-well plates with Milli-Q water in the correct diluent volumes for the 1:10 and 1:100 plates.
3. Perform the dilution process with these plates.
4. Confirm that the transfer is even across the plate (e.g. tips are pressed on properly) and that the well heights are correctly adjusted to ensure aspiration, dispensing, and mixing.

Dye-based testing of dilution process

Once it is confirmed that your dilution process is set up properly, we recommend testing with a dye, such as crystal violet or fluorescein, to verify proper mixing. It is important to test at least two sequential steps in the serial dilution process as this is the easiest way to identify insufficient mixing on the intermediate plate.

1. Pre-dilute the testing dye.
    a. If using crystal violet dye, dilute the stock crystal violet 1:10 in Milli-Q water in a 15 mL conical tube and mix by vortexing.

    b. If using fluorescein, dilute to 25 µg/mL in Milli-Q water in a 15 mL conical tube and mix by vortexing.

2. Label a 96-well plate “undiluted sample”. Pour the pre-diluted dye into a reservoir. With a multichannel pipettor, aliquot the same volume of dye as you would sample onto the undiluted sample plate.
3. Label two 96-well plates “1:10” and “1:100”. Pour Milli-Q water into a reservoir. With a multichannel pipettor, aliquot the same volume of Milli-Q water as diluent for the 1:10 and 1:100 plates.
4. Perform the dilution process with these plates. Use a clean box of pipette tips for each transfer.
5. Transfer the liquid from the 1:100 plate into a flat bottom plate. Measure on a plate reader (crystal violet: absorbance - 570 nm; fluorescein: excitation - 498 nm, emission - 517 nm).
6. Compare the absorbance or fluorescence across the plate and with that of a 1:100 dilution of the pre-diluted dye performed using a single pipettor.

If the mixing is insufficient on the 1:10 plate, then the transfer to the 1:100 plate will be at varying concentrations resulting in different amounts of dilution in different wells. To remedy this issue, we recommend including multi-height mixing, increasing the pipetting speed, and/or increasing the number of mixing cycles.

Dye-based testing for splashing or aerosolization

Once the mixing is sufficient, it is important to ensure that no splashing or excess aerosolization is occurring. To this end, perform a “checkerboard test” using undiluted crystal violet dye or 25 µg/mL of fluorescein dye.

1. Fill every other well of a 96-well plate with dye and the remaining wells with Milli-Q water in a checkerboard pattern (plate A). The volume in each well should be equal to the final volume of the dilution plates.
2. Make an additional 96-well plate with the same checkerboard pattern (plate B).
3. Perform the transfer and mixing steps of the dilution process going from plate A to plate B and then back to plate A. Complete this 10 times.
4. Transfer the liquid from plate A into a flat bottom plate. Measure on a plate reader (crystal violet: absorbance - 570 nm; fluorescein: excitation - 498 nm, emission - 517 nm).
5. Measure the background of clean Milli-Q water on the plate reader.

The goal is for the wells with Milli-Q water on plate A to be indistinguishable from the clean Milli-Q water, suggesting that the mixing protocol is not splashing or aerosolizing the concentrated samples.

NIST positive PCR control-based testing

Once the dilution process passes dye-based testing, perform a similar test with a NIST positive PCR control. This test serves two functions: (1) confirmation that the amount of dilution is correct and (2) confirmation that there is no well-to-well contamination. This is important because 16S rRNA qPCR and ddPCR are both more accurate and sensitive than a plate reader.

NOTE: Perform Steps 1-5 of this test in the PCR workstation.

1. Pre-dilute the NIST positive PCR control with nuclease-free water as needed depending on the chosen PCR method and your dilution process setup. The final dilution of NIST positive PCR control after Step 3 should be $1{:}10^3$ for qPCR or $1{:}10^4$ for ddPCR. Follow a process for serial dilution analogous to the one described in Reagent setup for the standard plasmid serial dilution.
2. Fill every other well of a 96-well plate with pre-diluted NIST positive PCR control and the remaining wells with nuclease-free water in a checkerboard pattern. It is acceptable for this to be a partial plate (e.g. 1/3 filled) if prior testing indicated consistency across the plate. Leave space for the additions in Step 4 and, if performing qPCR, the standard curve dilution points. This plate serves as the undiluted sample plate.
3. Setup and perform at least two serial dilution steps from your dilution process.
4. Take the resulting sample dilution plate and add multiple wells of the same NIST positive PCR control serially diluted to $1{:}10^3$ for qPCR or $1{:}10^4$ for ddPCR using a single pipettor. Follow a process for serial dilution analogous to the one described in Reagent setup for the standard plasmid serial dilution.
5. Add multiple wells of nuclease-free water (e.g. NTC). This is the final sample dilution plate for the subsequent qPCR or ddPCR.
6. Resume the main protocol from Step 49.
7. Confirm that the resulting measurements of NIST positive PCR control are consistent across wells in the checkerboard. Further confirm that the resulting measurements are consistent between the NIST positive PCR control wells diluted with the dilution process and diluted with a single pipettor. Then evaluate that the measured 16S rRNA copies per µL is similar to the expected value for that NIST positive PCR control. See the main protocol analysis sections for the specific calculations.
8. Check that none of the NTC wells are contaminated by the concentrated NIST positive PCR control in adjacent wells of the checkerboard. Specifically, the NTC wells across the checkerboard should result in similar 16S rRNA copies per reaction. Furthermore, these values should be similar to the 16S rRNA copies per reaction for the NTC wells added in Step 5.

If there are discrepancies in Step 7, further evaluate the dilution process and consider increasing the amount of mixing. If contamination is occurring according to Step 8, reduce the vigor of mixing or otherwise modify the process to reduce contamination.

Supplementary Methods 4 – Standard curve testing

The purpose of Supplementary Methods 4 is to ensure accuracy and precision in the serial dilution of the standard curve plasmids. There are two main ways to assess this. First, we suggest performing three serial dilutions of each of the two stock plasmids and assaying them together on a test plate to assess precision. All six serial dilutions should be similar. Second, we suggest including NIST positive PCR controls alongside NTCs in this testing to assess the accuracy of the resulting standard curve (e.g. linear regression).

NOTE: Perform Steps 1-4 of Supplementary Methods 4 in the PCR workstation.

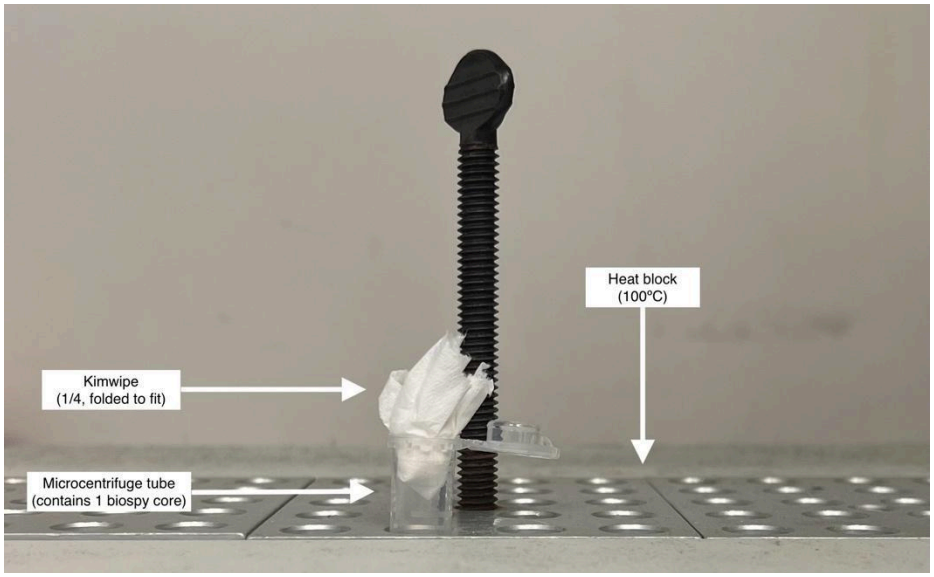
1. Prepare three serial dilutions of the *P. vulgatus* and *F. prausnitzii* plasmids as described in Reagent setup in the main protocol.
2. Prepare two 1:10 serial dilutions out to $1:10^3$ of the NIST positive PCR control. Follow a process for serial dilution analogous to the one described in Reagent setup for the standard plasmid serial dilution.

   NOTE: NIST does not recommend storing diluted samples, so these must be diluted each day.
3. Create a 96-well plate layout including three NTCs, the two $1:10^3$ diluted NIST positive PCR controls, and each of the serial dilutions of *P. vulgatus* and *F. prausnitzii* from $1:10^3$ to $1:10^9$.
4. Aliquot the volume calculated in Step 49Ai of NTCs, NIST positive PCR controls, and standard curve dilution points to the corresponding wells on a 96-well plate. This plate should be treated as the final sample dilution plate hereafter.
5. Follow Steps 49Aiii-49Axxii of the main protocol to perform 16S rRNA qPCR.
6. Analyze the resulting data to assess the limit of blank as defined by the NTCs, the consistency amongst the six serial dilutions, and the accuracy (e.g. measured / expected) of the 16S rRNA copies per µL for the NIST positive PCR controls. For more details on the analysis, refer to qPCR-specific analysis (Steps 49Axxiii-49Axxxixi) and Shared analysis and quality control (Steps 50-65) in the main protocol.

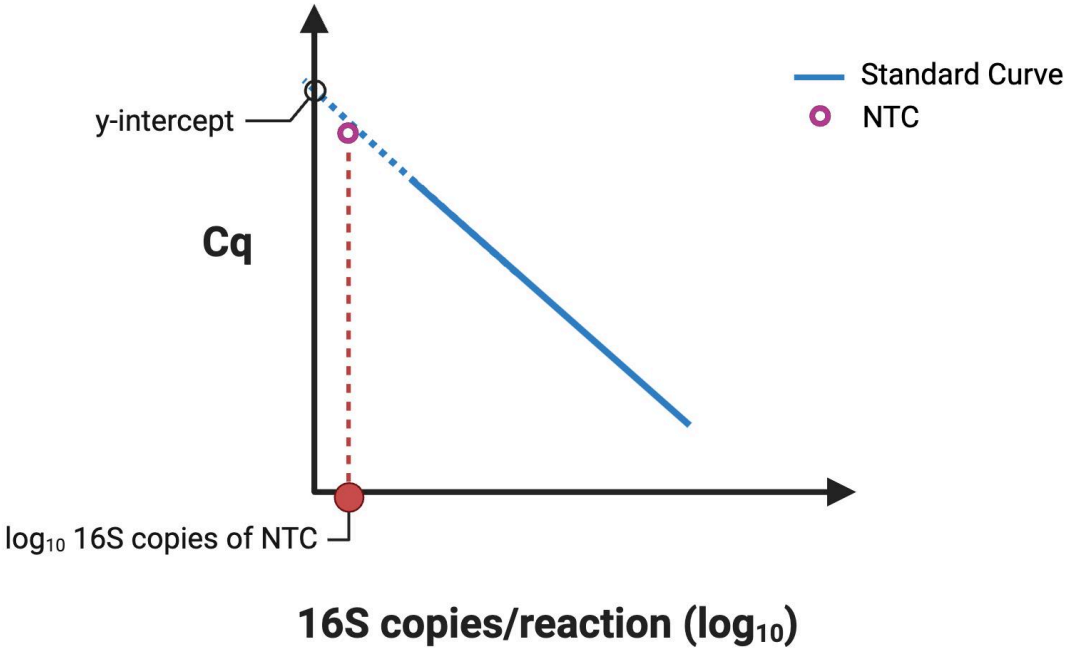
Once the serial dilutions of the standard curve plasmids pass testing, proceed with one serial dilution from each of *P. vulgatus* and *F. prausnitzii* for subsequent qPCR assays.

New batches of standard curve serial dilutions should be tested in a similar way. It is not always necessary to prepare them in triplicate, but we do recommend always analyzing the test data following Steps 49Axxvii-49Axxxii for the standard curve and Steps 50-51 for the NIST positive PCR controls.

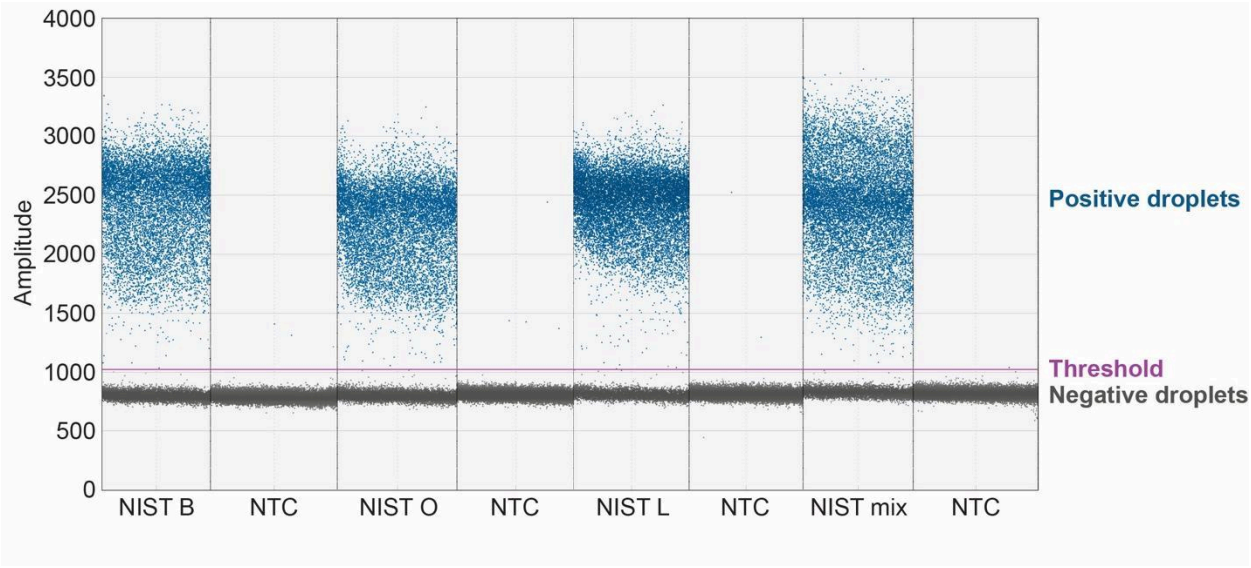
Supplementary Figures



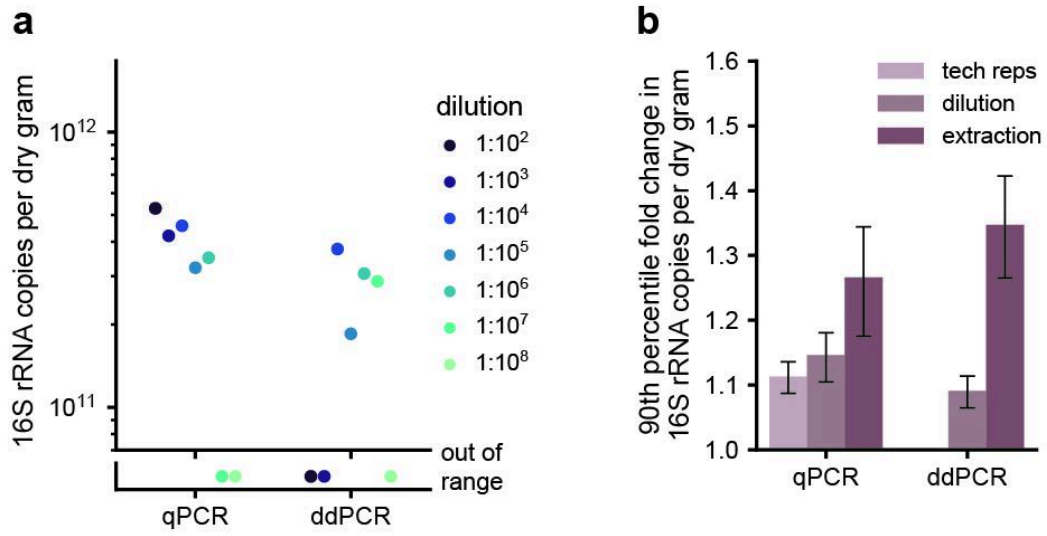
Supplementary Figure 1 Measurement of stool sample moisture content setup. Photograph of stool moisture content setup (Steps 30-31) of the main protocol.



Supplementary Figure 2 Calculating 16S rRNA copies per reaction in qPCR. Illustrated graph depicting Step 49Axxxiv of the main protocol. The Cq value of the NTC from Step 49Axxiv is converted to 16S rRNA copies per reaction using the linear regression line of the standard plasmids from Step 49Axxxii. The calculated value for the limit of blank should be 500 or less 16S rRNA copies per reaction.



Supplementary Figure 3 Thresholding for 16S rRNA ddPCR. Fluorescence intensity plots depicted for controls in the 16S rRNA qPCR and ddPCR comparison experiment. Each dot represents a single droplet in a given reaction. Columns represent either a NIST positive PCR control component, the NIST mixture as described in Reagent setup, or an NTC. The threshold is set based upon the NIST positive PCR controls and NTCs. The negative droplet cluster should be consistent across all wells.



Supplementary Figure 4 16S rRNA qPCR and ddPCR comparison (copies per dry gram). Informed consent was obtained from all donors (Stanford IRB 42053). **a**, Comparison of a single high abundance stool sample assayed at multiple dilutions in qPCR and ddPCR. Abundance is reported as 16S rRNA copies per dry gram of stool. Each data point represents one serial dilution point of the same sample; the data point color indicates the amount of dilution. Dilutions

that fall outside of the quantification range are shown below the plot. **b**, Assessment of variability at different stages of the measurement process in qPCR and ddPCR. The abundance measurement is reported as 16S rRNA copies per dry gram of stool. The technical replicate bar is calculated from qPCR technical replicates (3 replicates, n=8 samples). The dilution bars are calculated by diluting and assaying the same extracted DNA three times (n=8 samples). The extraction bars are calculated by biopsy punching, extracting, diluting, and assaying the same stool sample three times (n=8 samples). Error bars indicate 10th and 90th percentiles from bootstrapping. The y-axis is the fold change of the 90th percentile compared to the mean based upon the standard deviation of the three replicates.