Poster #1031

Quantitative Whole Metagenomic Sequencing (qWMS): a Powerful Tool for Advancing Our Understanding of the Microbiome and its Impact on Health and Disease.

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A quantitative and qualified method for untargeted and absolute profiling of microbiome samples will provide an essential tool for microbiome drug development.

The microbiome field has relied on whole metagenomic sequencing (WMS) to profile microbial species, but it has two major limitations. First, WMS produces relative abundance (%RA) measurements, focusing on the percentage each species contributes to the community rather than quantifying the population size (cells/mg of stool). This makes interpreting %RA data challenging, as expansion of one taxon may not be distinguishable from a contraction of others. Second, biases incurred during sample collection, DNA extraction, and bioinformatic analysis can distort microbiome profiles. Drug development requires reproducible, quantitative assays to evaluate both drug pharmacology and how a drug may impact the gastrointestinal microbiome; establishing WMS as a quantitative method to determine the abundance of microbes in the GI tract will advance these efforts.

We developed a quantitative WMS (qWMS) assay to convert %RA data into absolute values (cells/mg of stool) using a spiked non-commensal environmental Cell Reference Standard (CRS), *Pseudoalteromonas piscicida*, of known titer (1E6 cells) prior to DNA extraction. We confirmed that maintaining the CRS above 0.02% of the community ensures accurate and precise absolute measurements, across a variety of stool backgrounds. Assay linearity (R^2=0.99) was confirmed using 64 spiked samples across multiple logarithmic scales. Using mixed microbial cell reference standards (three Gram-positive and three Gram-negative) spiked into a representative stool matrix; we observed limited extraction bias, high assay precision (<16% CV), and good accuracy (%error= 3-54%).

We then applied qWMS to a set of stem-cell transplant patient samples (n=334), where domination (>30% relative abundance) of certain pathobionts in stool has been linked to higher post-transplant mortality risk (Stein-Thoeringer et al. 2019). We validated our qWMS findings by correlating them with data from four pathobiont-specific qPCR assays (R^2=0.89-0.98). The high concordance between methods supports the use of this assay for accurately quantifying microbial species in patient samples. Absolute species measurements enhance patient microbiome data interpretation, clarify disease associations, refine drug development targets, and boost biomarker identification. This transformative tool overcomes the limitations of traditional %RA data, advancing microbiome research and addressing the reproducibility crisis head-on through rigorous vetting of its quantitative accuracy and precision.

Relative data provide a limited view of species abundance, and pipeline differences can bias results.

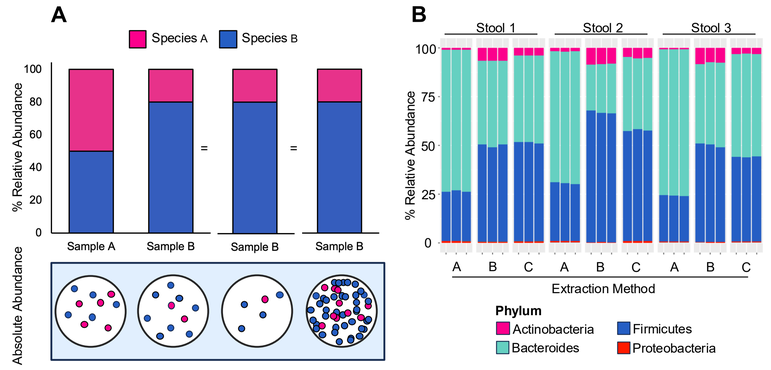
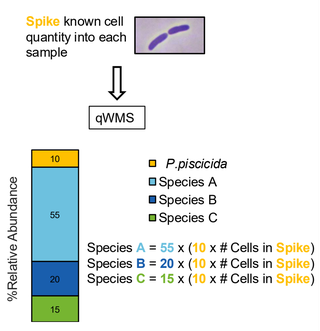


Figure: (A) Depiction of relative vs. absolute abundance of two species. Between samples, relative abundance cannot determine whether a species is more or less abundant, or the degree of change in abundance. **(B)** Relative abundance of phyla in stool samples. Three different DNA extraction methods yield different %RA profiles from the same materials.

Quantitative WMS (qWMS) provides absolute abundance measurements relative to a spiked reference species.

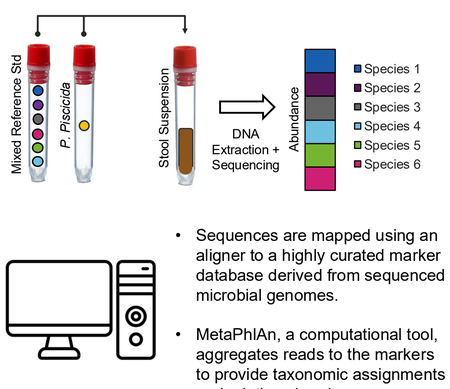
Cell Reference Standard (CRS): ***Pseudoalteromonas piscicida***

- Marine seawater isolate not found in human microbiome samples
- Easily identifiable cells in groups of 1 or 2
- Good agreement between orthogonal methods of quantification
- Added to all samples



Mixed Cell Reference Standard (MCRS)

- Equal mixture of 6 microbiome species, 3 Gram-positive and 3 Gram-negative
- Added at target amount to a single lot of donor stool which does not contain these organisms, along with Cell Reference Standard
- Entire mixture is extracted and sequenced



- Sequences are mapped using an aligner to a highly curated marker database derived from sequenced microbial genomes.
- MetaPhlAn, a computational tool, aggregates reads to the markers to provide taxonomic assignments and relative abundance.

Citations:
C. K. Stein-Thoeringer et al. Lactose drives Enterococcus expansion to promote graft-versus-host disease. *Science* 366,1143-1149 (2019).
J. Kralj et al. Reference Material 8376 Microbial Pathogen DNA Standards for Detection and Identification. *NIST SP* 260-225. (2022).

Making and characterizing Cell Reference materials

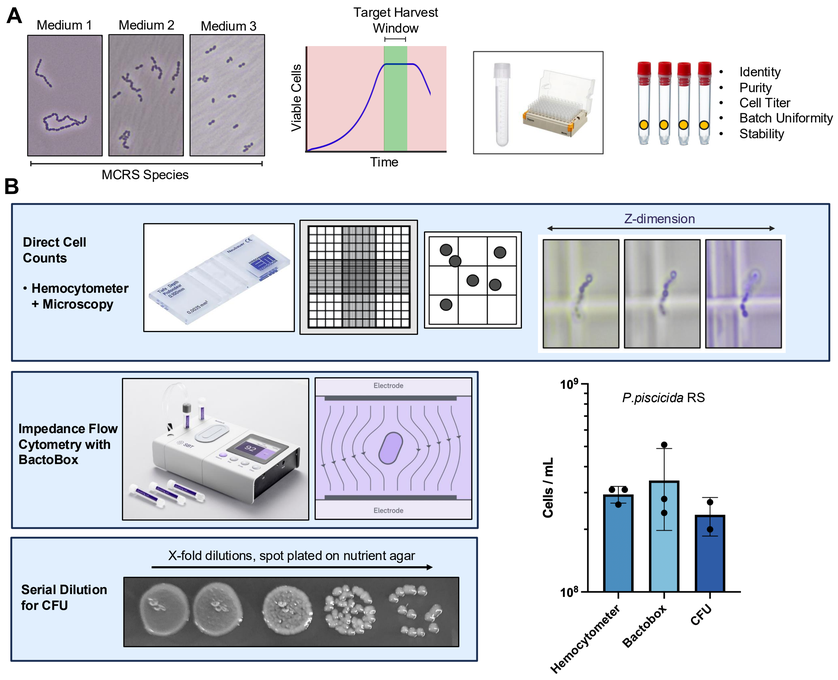


Figure: (A) Process for making Cell Reference materials. Media screening and pilot studies to identify conditions for capturing single cells in stationary phase onset. Growth, harvest, vialing, and storage of lots. Quality attributes. **(B)** Cell quantification by orthogonal methods. Hemocytometer was chosen as value to mix on.

qWMS is reproducible if CRS is > 0.02% of population.

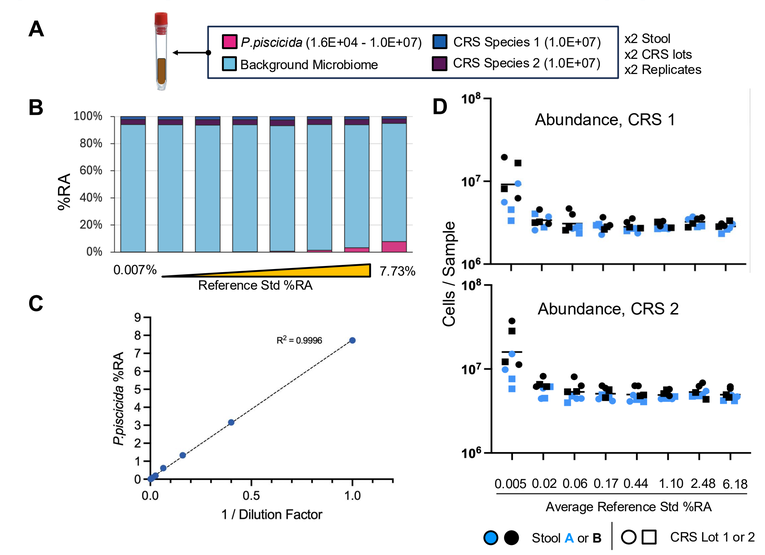


Figure: (A) Experimental Design. Dilution series (2.5X-fold) of two *P.piscicida* CRS lots spiked into two donor stools. Two other CRS spiked into every sample at a constant level. **(B)** Relative abundance of spiked species and background microbiome in a representative series (CRS lot 1, Stool A, Replicate 2). **(C)** *P.piscicida* %RA as a function of dilution factor with line of best fit for samples in (B). **(D)** Absolute abundance of other Reference Species calculated from *P.piscicida* %RA within each sample.

qWMS is precise, accurate, and repeatable.

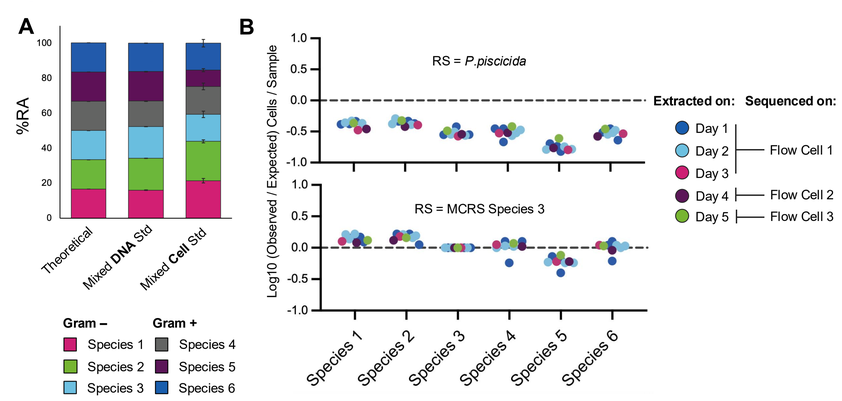


Figure: (A) Relative abundance of DNA and cell-based reference standards (equal genome copy # and cell #, respectively). **(B)** Absolute abundance of species in Mixed Cell RS, relative to expected, colored by extraction date and sequencing run. *P.piscicida* may underestimate absolute abundance of other species. Accuracy is further improved by another CRS or application of a scaling factor. Top panel: *P.piscicida* is RS. Bottom panel: Species 3 is RS.

qWMS pipeline is automated and integrated with dry weight collection to normalize for sample mass.

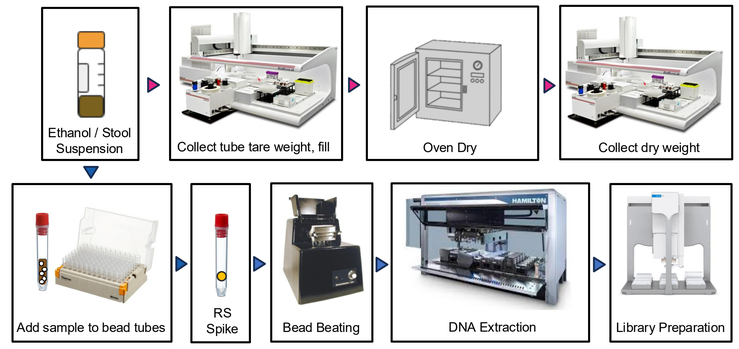


Figure: Samples are split for automated and parallel dry weight collection (top) and DNA extraction, library preparation (bottom).

Absolute abundance measurements by qWMS and qPCR are strongly correlated in patient samples.

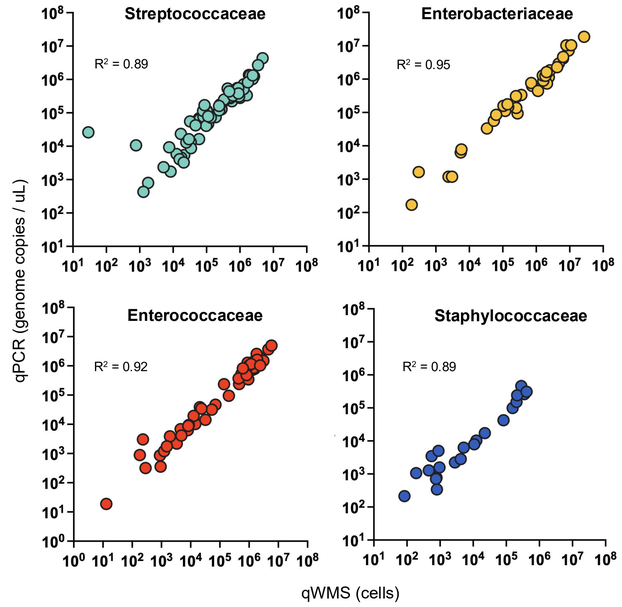


Figure: qWMS benchmarked against qPCR in stool samples from a cohort of patients undergoing stem-cell transplantation. Replicate stool samples analyzed via qWMS or qPCR. There are samples where qWMS value = 0 due to lower sensitivity not shown in each plot (clockwise from top left, 7, 34, 49, 22 samples)

Pathobiont domination (>30% RA) does not necessarily correspond with high pathogen abundance.

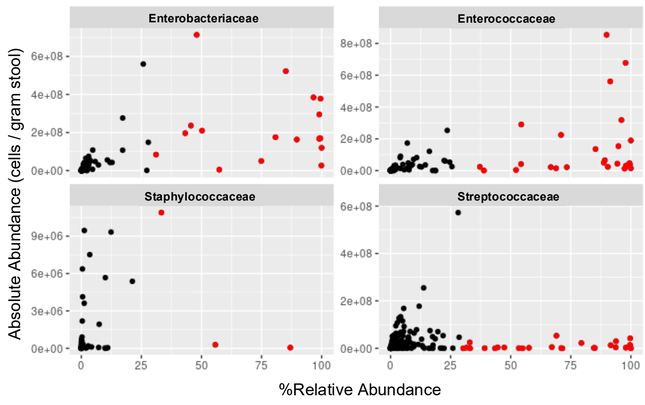


Figure: Absolute abundance of pathobionts (qWMS) as a function of relative abundance in patient samples. Red points indicate samples where family %RA = >30% of the entire sample, a threshold for community domination in this population (Stein-Thoeringer et al. 2019).

Conclusions:

- The qWMS assay and controls provide a highly automated method for accurately quantifying species in patient samples in absolute terms.
- Assay performance was vetted using internally-developed and characterized reference standards.
- Absolute abundance can provide an alternative view of preclinical and clinical data that support drug development, including drug pharmacokinetics and species – host interactions.