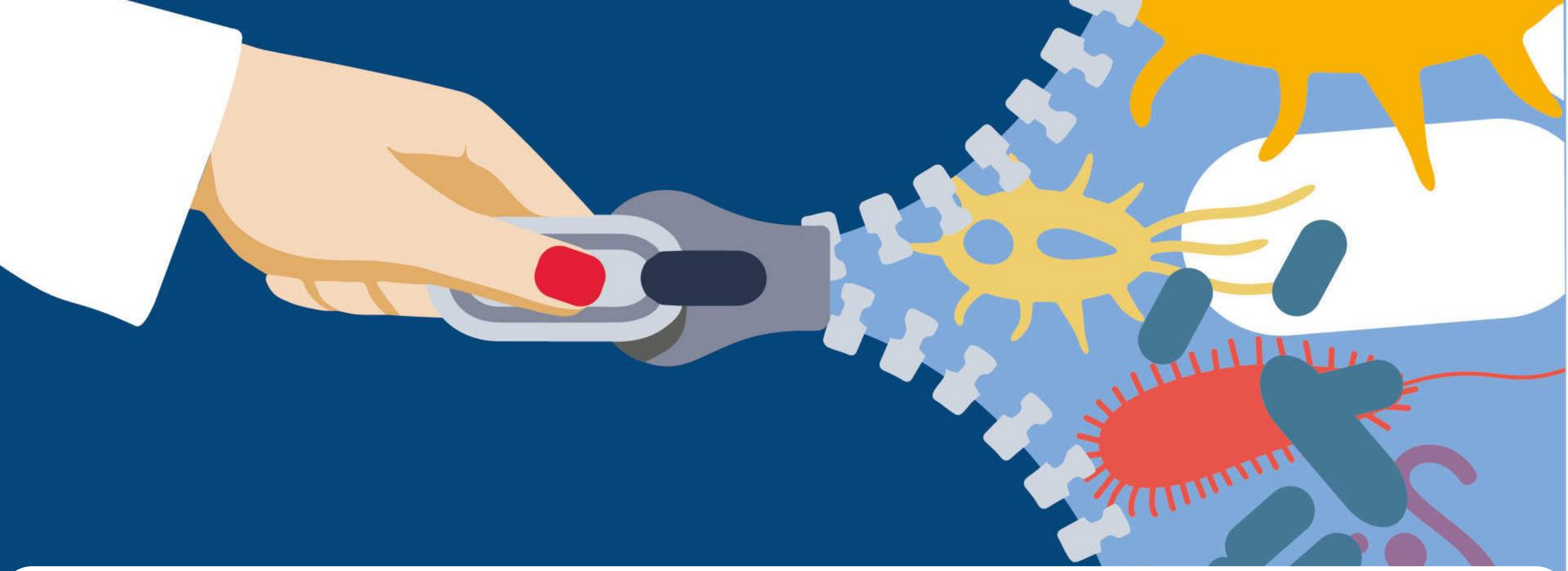


Challenges of sequencing low-biomass samples

Dominic O’Neil, R&D Director, Microbiome Product Development

Legal disclaimer

QIAGEN products shown here are intended for molecular biology applications. These products are not intended for the diagnosis, prevention or treatment of a disease.

For up-to-date licensing information and product-specific disclaimers, see the respective QIAGEN kit instructions for use or user operator manual. QIAGEN instructions for use and user manuals are available at **www.qiagen.com** or can be requested from QIAGEN Technical Services (or your local distributor).

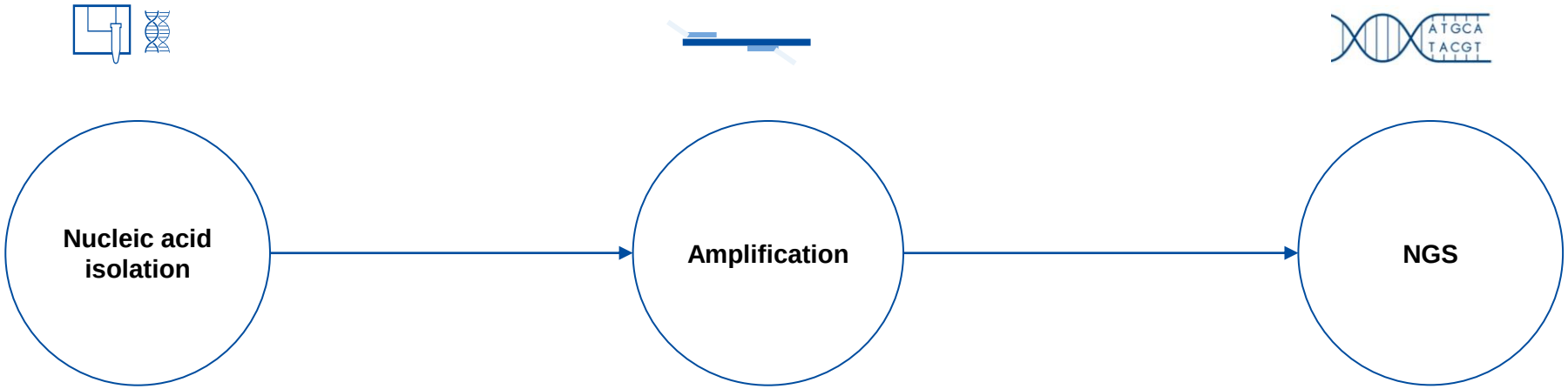
Defining low-biomass

- **People mean different things when they talk about low-biomass**
  - low total amount of DNA in a sample – is it ng, pg, or fg? Those all have different challenges
    - Rough categories: 1-100 ng as low, 1-100 pg as ultra-low, and fg as single-cell
  - low microbial load in a background of other DNA
- **Challenges of sequence amplification are different than those of WGS**
  - Current technologies allow consistent amplification-free library construction and sequencing of samples in the ng range
  - Standard amplification (e.g. 16S PCR) allows analysis of samples in the pg range
  - Single-cell techniques (e.g. RepliG MDA) most likely needed for fg ranges

Topics

1. Ultra-clean reagents for extraction and amplification
2. Metagenomic study including low-biomass samples (MASTER)

QIAGEN offers a comprehensive portfolio of ultra-clean solutions



- QIAamp DNA Microbiome Kit
- DNeasy PowerSoil Pro*
- QIAamp UCP DNA Micro Kit
- RNeasy UCP Micro Kit
- QIAamp UCP Pathogen Mini Kit
- QIAamp DNA FFPE Advanced Kit
- QIAamp MinElute ccfDNA Mini/Midi Kits
- miRNeasy Tissue/Cells Adv. Micro Kit
- miRNeasy Serum/Plasma Advanced Kit
- MagAttract kits

Digital PCR

- QIAcuity UCP Probe PCR Kit

PCR

- UCP Multiplex PCR
- UCP Probe PCR

MDA

- RepliG Single Cell Kit

Reagents

- TaqNova Polymerase UCP (200 U)
- Microbial DNA-Free Water
- UCP PCR Water

Microbial detection

- QIAseq xHYB Viral and Bacterial Panels

16S rRNA and ITS profiling

- QIAseq 16S/ITS Panels

Metagenomic shotgun sequencing

- QIAseq FX DNA Library Prep

Metatranscriptomics

- QIAseq FastSelect 5S/16S/23S
- QIAseq Stranded Total RNA Library

Nucleic Acid Extraction Kits

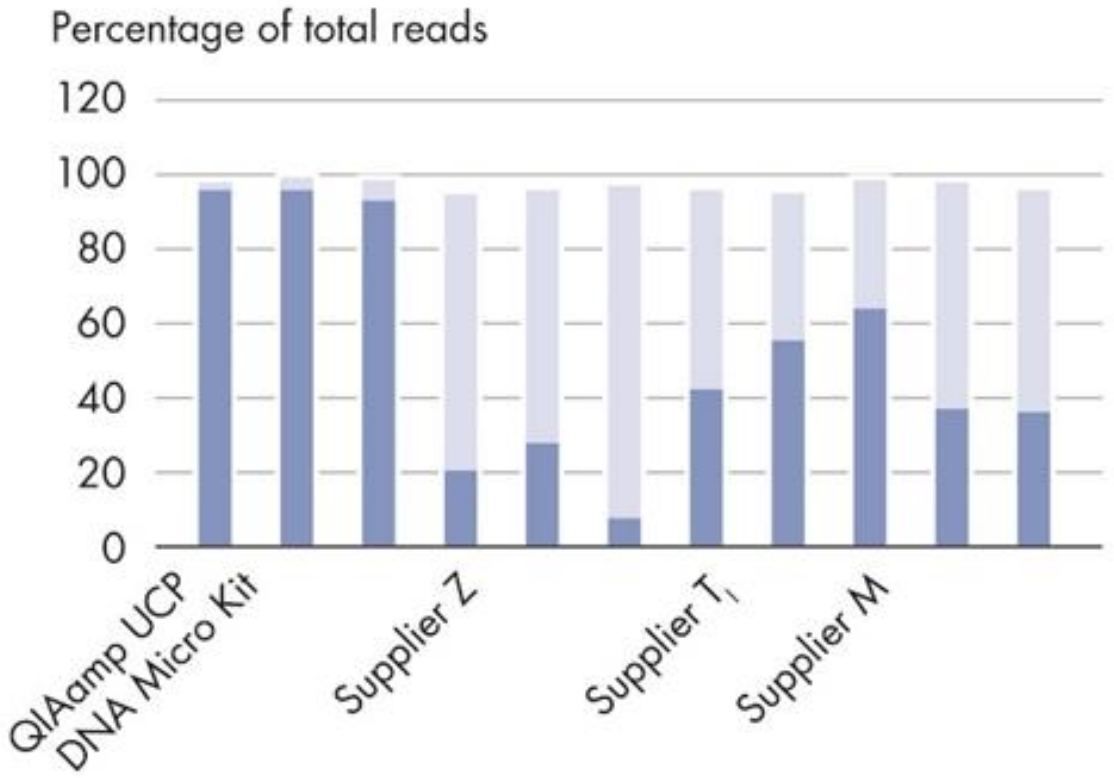
Many different components go into a kit

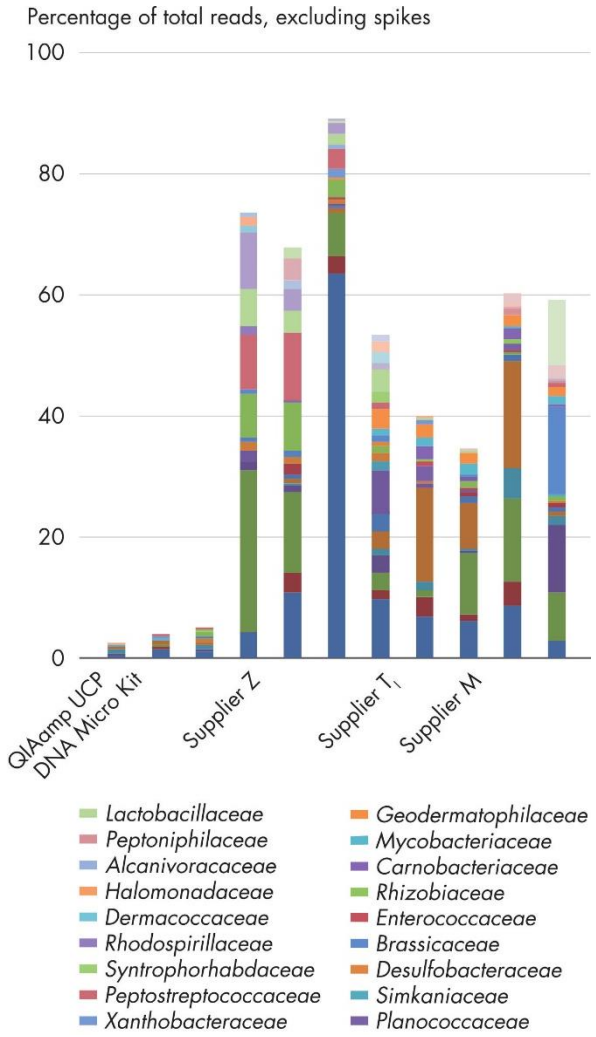
Component	Risk	Mitigation
Water	Medium	Cleaning system in place, chemical or physical treatment
Binding Substrate (Membrane/Beads)	Medium	Chemical or physical treatment
Plastics	Low	Treatment
Raw Chemicals	Varies	Supplier validation, chemical or physical treatment, purification after mixing
Enzymes	High	Purification, chemical treatment
Handling (Mixing, Filling, Packing, etc)	Low	Good Processes

QC testing imperative to verify treatment effectiveness and compliance with spec!

Treatment of extraction kits reduces background

QIAamp UCP DNA Micro Kit





Results indicate background of 200-1000 16S copies in non-specialized kits

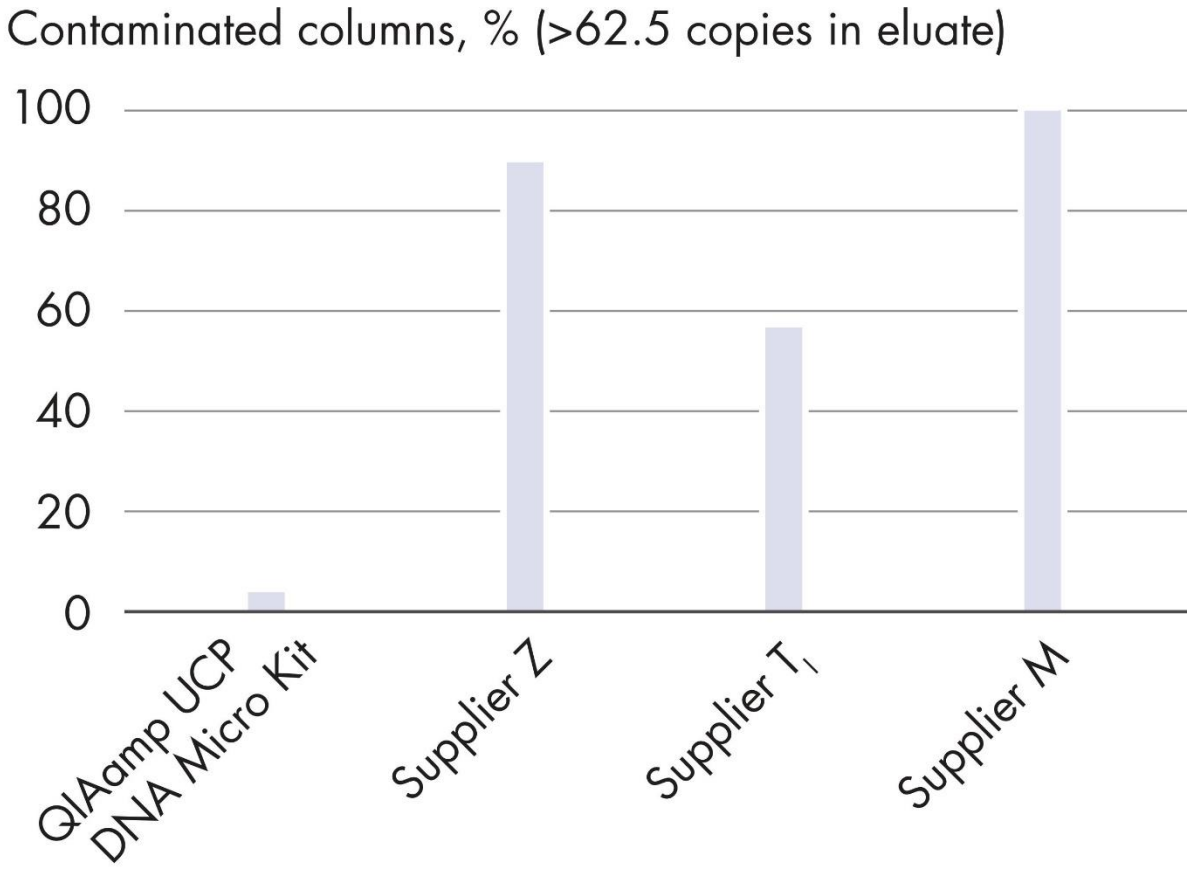
Background < 20 16S copies in treated kit

200 copies of bacterial DNA (dark blue) were spiked into eluates.

16S sequencing on a MiSeq

Light blue bars indicate contamination

Treatment of extraction kits reduces background



30 extractions from a clean kit, 16S amplification with a cleaned PCR mix

Cutoff for contamination (2.5 copies/µl) determined by NTC in PCR and lowest detectable dilution

Even in a clean kit, some examples may show contamination. Possibly environmental.

Examples shown are column-based kits, but Mag-bead based methods are also susceptible and must be controlled!

QC Examples from CoAs

DNeasy PowerSoil Pro

Test parameter	Test description	Acceptance criteria
Conductivity Solution CD1	Conductivity 1:10 (mS/cm)	9.46 – 11.39
Conductivity Solution CD2	Conductivity 1:10 (mS/cm)	24.81 – 29.66
pH Solution CD3	pH	6.80 – 7.20
Conductivity Solution CD3	Conductivity 1:10 (mS/cm)	33.21 – 39.98
pH Solution C5	pH	7.40 – 7.90
Conductivity Solution C5	Conductivity 1:2 (mS/cm)	2.71 – 3.13
pH Solution C6	pH	8.00 – 8.50
Absence of bacterial DNA on membrane MB Spin Columns	Panbacterial realtime PCR detecting 16S rDNA sequences	≤ 10 copies of *E. coli* genome or equivalent per µl of eluate

QIAamp UCP DNA Micro

Quality Control Passe

Absence of bacterial DNA

Kit materials and reagents were tested for the absence of bacterial DNA in a functional kit test using pure water samples and elution in 25µl elution buffer.

Absence of bacterial DNA in the eluates of the functional kit test was confirmed using a panbacterial realtime PCR detecting 16S rDNA sequences.

No bacterial DNA could be detected down to a limit of detection of <50 fg/µl in the eluate.

Buffers

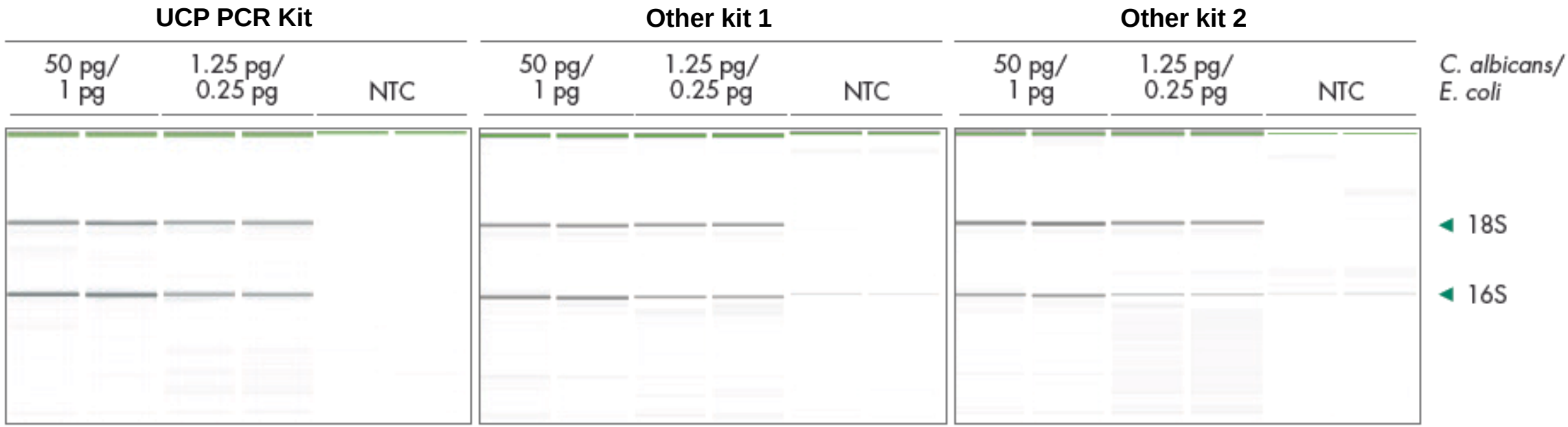
Conductivity and pH of buffers and solutions were tested and found to be within the ranges below:

	Conductivity (mS/cm)	pH
Buffer AUT	20.50–28.20	8.10–8.50
Buffer AUL	38.40–45.40 (1/10 dilution)	5.90–6.10
Buffer AUW1	50.20–59.80 (1/10 dilution)	–
Buffer AUW2	28.60–34.20	7.40–7.60
Buffer AUE	0.90–1.10	8.20–8.40

DNA background in the mix can bias results

Challenges

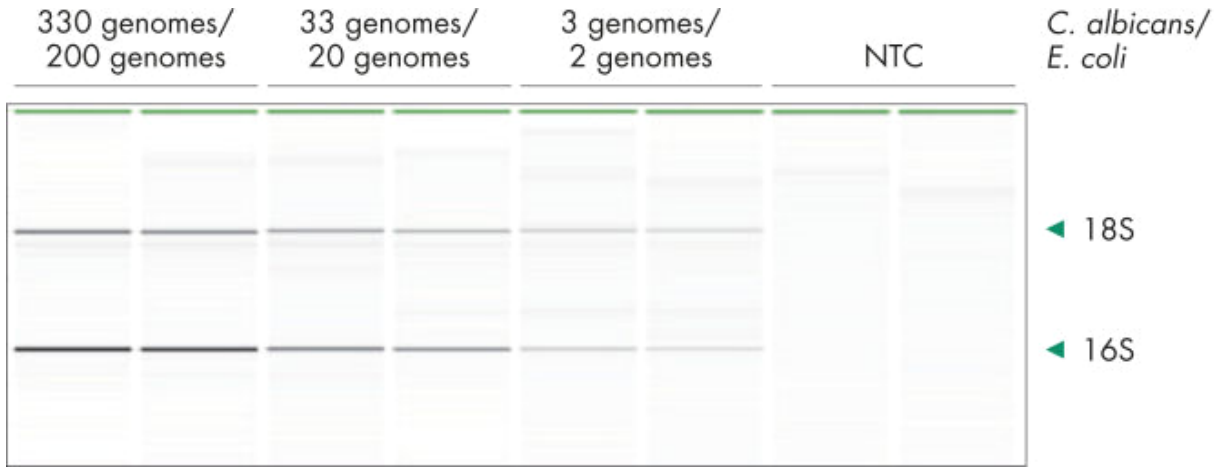
- **The PCR chemistry needs to amplify all bacterial 16S sequences evenly**
- **The PCR chemistry should not introduce any background**
- **The PCR chemistry needs to be sensitive enough for limited sample amounts**
- **The PCR chemistry should allow multiplexing of different targets and regions e.g., 16S regions plus ITS/18S**



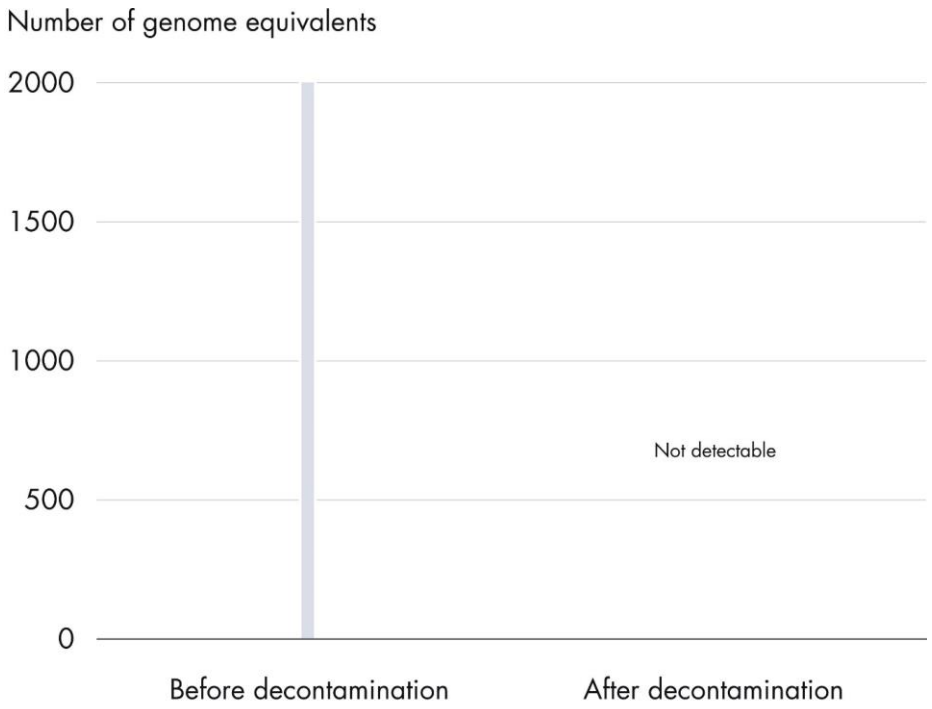
Treatment of amplification reagents

Amplification Reagents can be successfully treated to remove contaminating DNA, allowing detection of microbes at fg level

QIAGEN UCP Multiplex PCR Master Mix

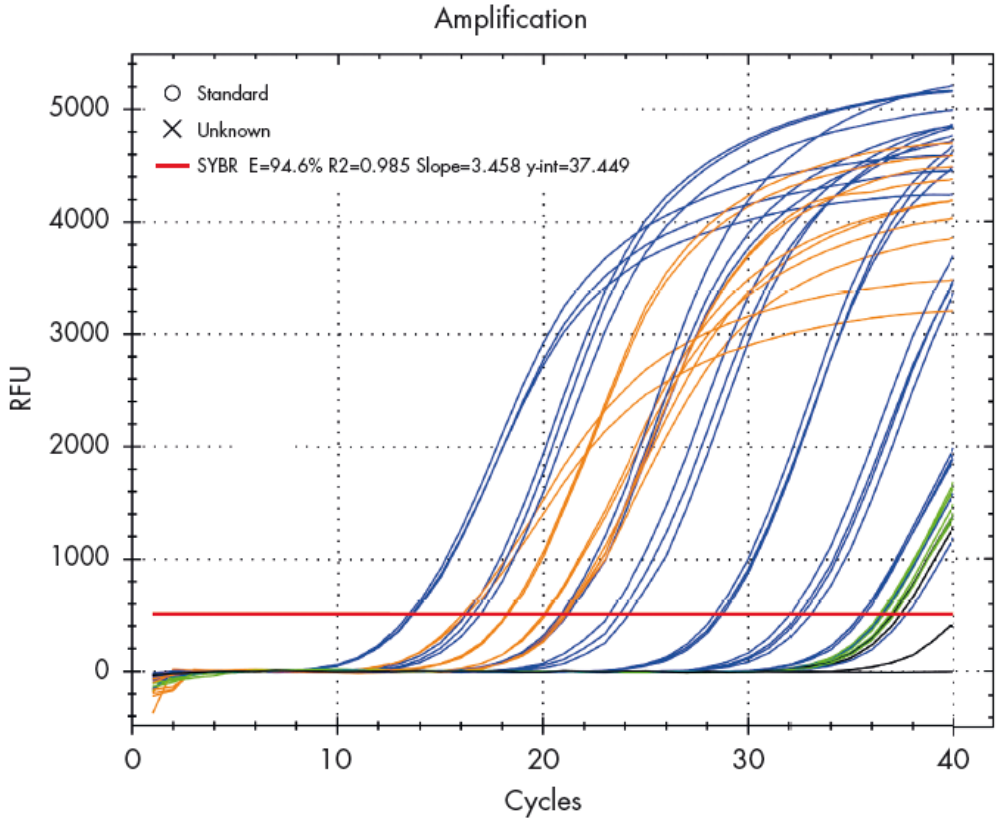


Repli-G Single Cell Kit

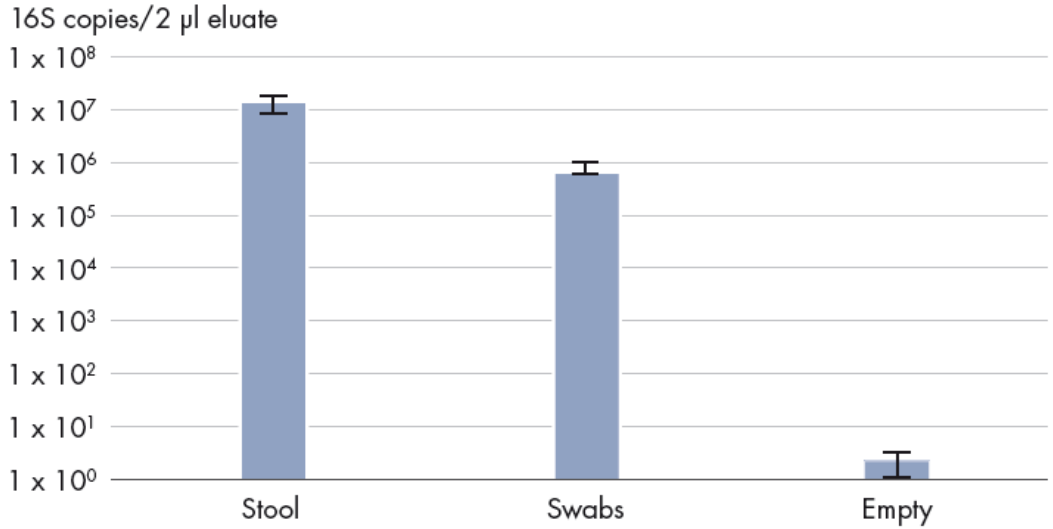


UCP Quant Kits: Precise determination of amplifiable DNA

16S DNA quantification shows high yields from samples and almost no kitome[1]



Blue: *E. coli* DNA standard dilution series from 10 ng/reaction to 10 fg/reaction. **Orange:** Buccal swabs; 2 µl of eluate used for quantification. **Green:** Empty FLOQSwabs. Results indicate a background signal of ~5 fg/µl eluate. **Black:** No-template control.



Quantification of buccal swabs and stool samples in one run. The bacterial loads show significant differences.

1) Note: Kitome: Background from the kit used

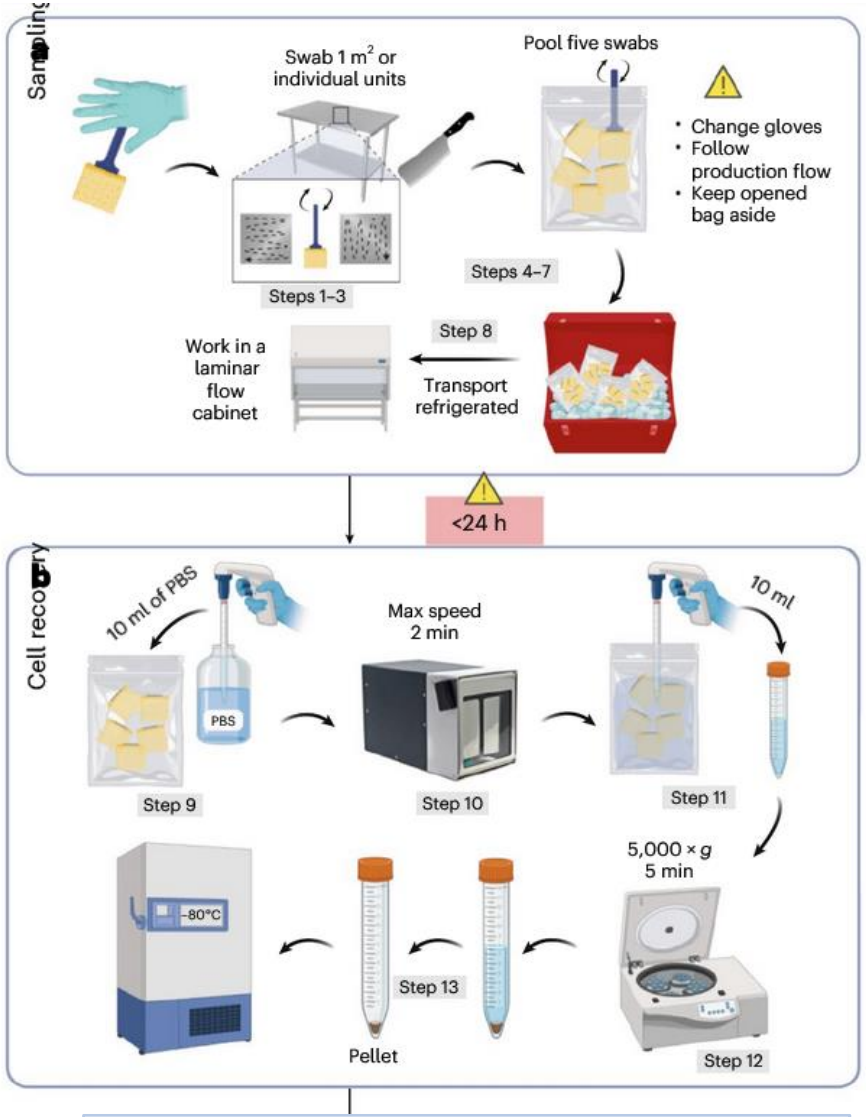
Metagenomic analysis is a system

All components should be considered, small variations in one area can introduce big shifts

- Sampling materials
- Sampling environment/s
- Storage and transport
- Extraction reagents
- Amplification reagents
- Sequencing reagents
- Laboratory environment/s
- Operator/s

Each individual component can be controlled to deliver undetectable levels of microbial background, but systems will often have additive effects leading to detectable background.

Example study – custom low-biomass kit for MASTER



DNA extraction with Dneasy PowerSoil Pro + UCP MinElute columns

- Study of microbiome in food and food processing facilities
- Many regularly cleaned, low-biomass surfaces (stainless steel tables, tools, etc)
- Also some high-biomass samples (e.g. food)

- **Development of custom QIAGEN extraction kit for low-biomass**

Negative Controls:

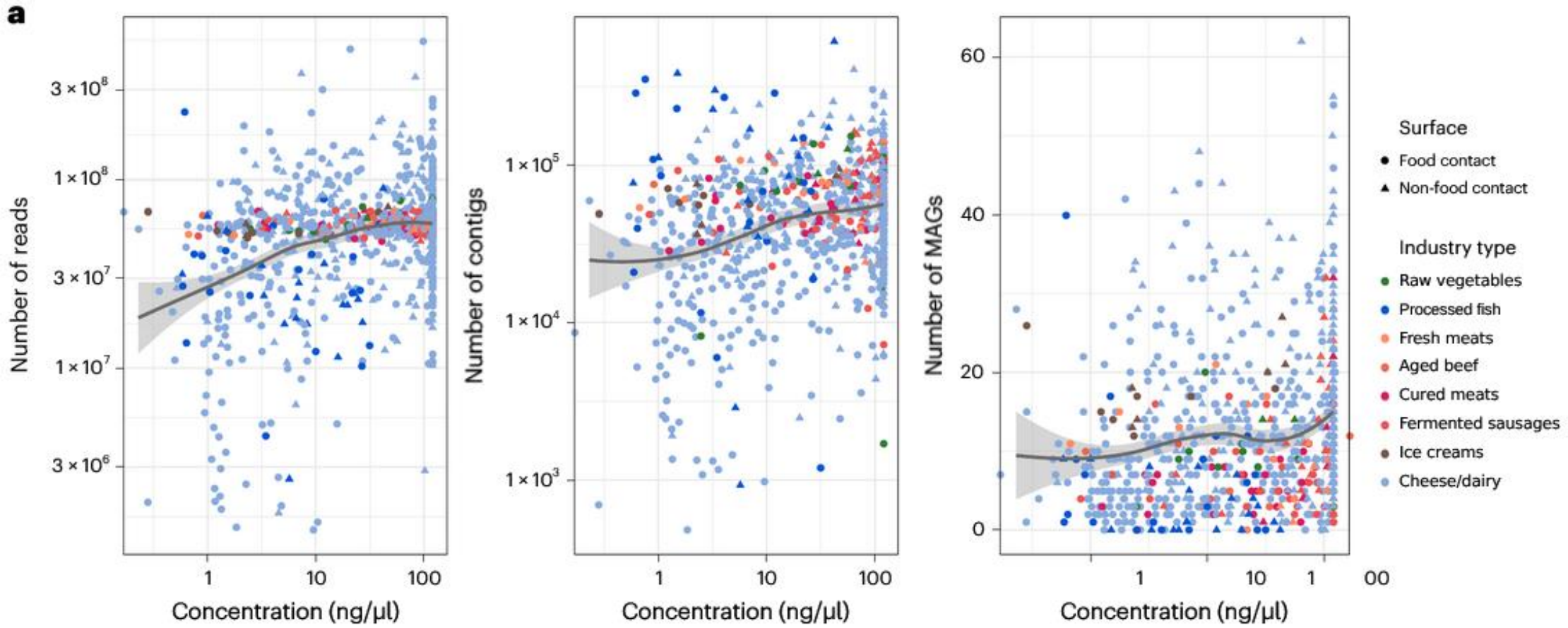
- **Swabs exposed to air at sampling site (neg. control industry) and at processing site (neg. control lab)**
  - Only 33.3% of these negative control samples could be sequenced, the vast majority of them with a low number of reads obtained.
- **Extraction negative control. All the negative controls from this category not detectable by Qubit high-sensitivity dsDNA quantification kit and failed in the library-preparation step**

This work was funded by the European Commission under the European Union's Horizon 2020 research and innovation program under grant agreement no. 818368 (MASTER).

Barcenilla, C., Cobo-Díaz, J.F., De Filippis, F. *et al.* Improved sampling and DNA extraction procedures for microbiome analysis in food-processing environments. *Nat Protoc* 19, 1291–1310 (2024). https://doi.org/10.1038/s41596-023-00949-x

Example study - MASTER

Effect of concentration on sequencing quality and information content



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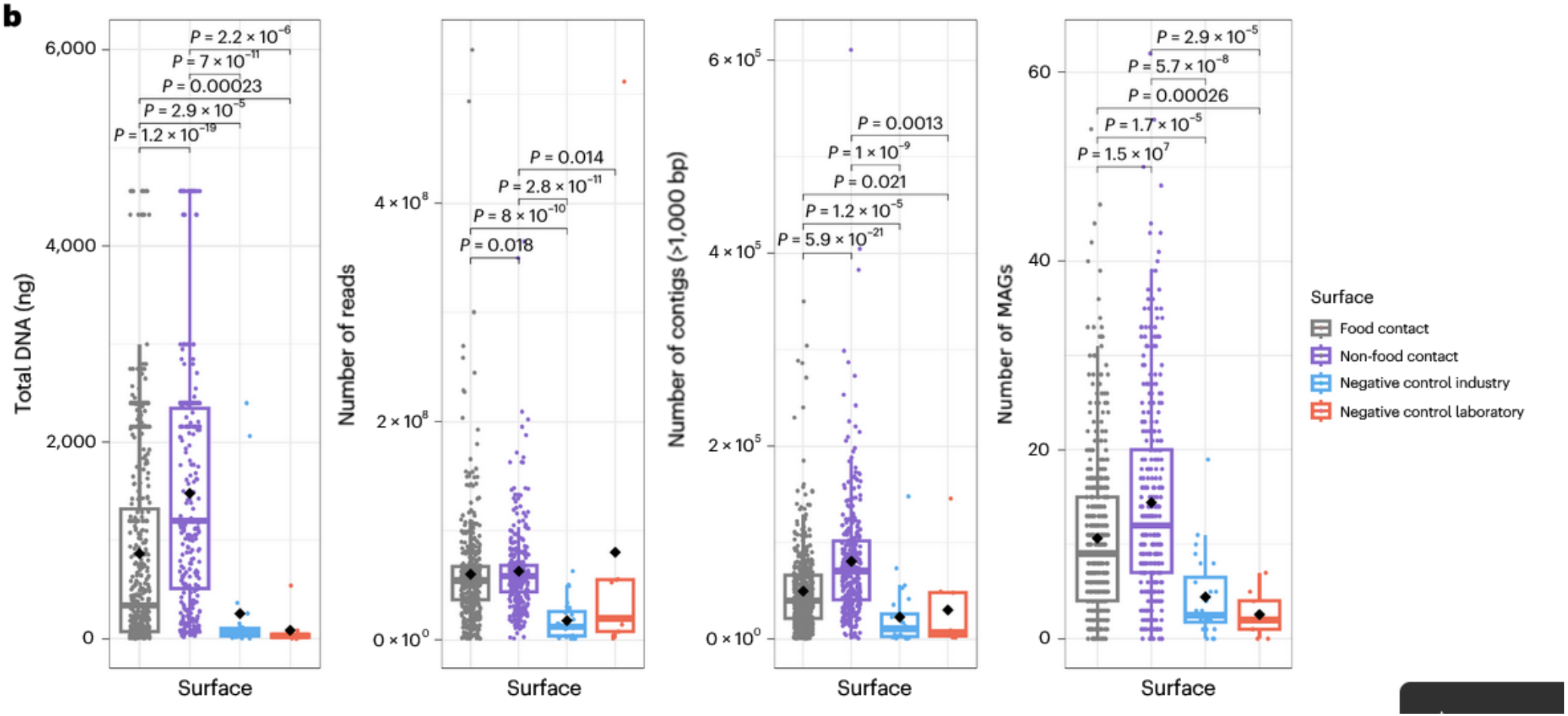
Example study - MASTER

Performance with negative controls



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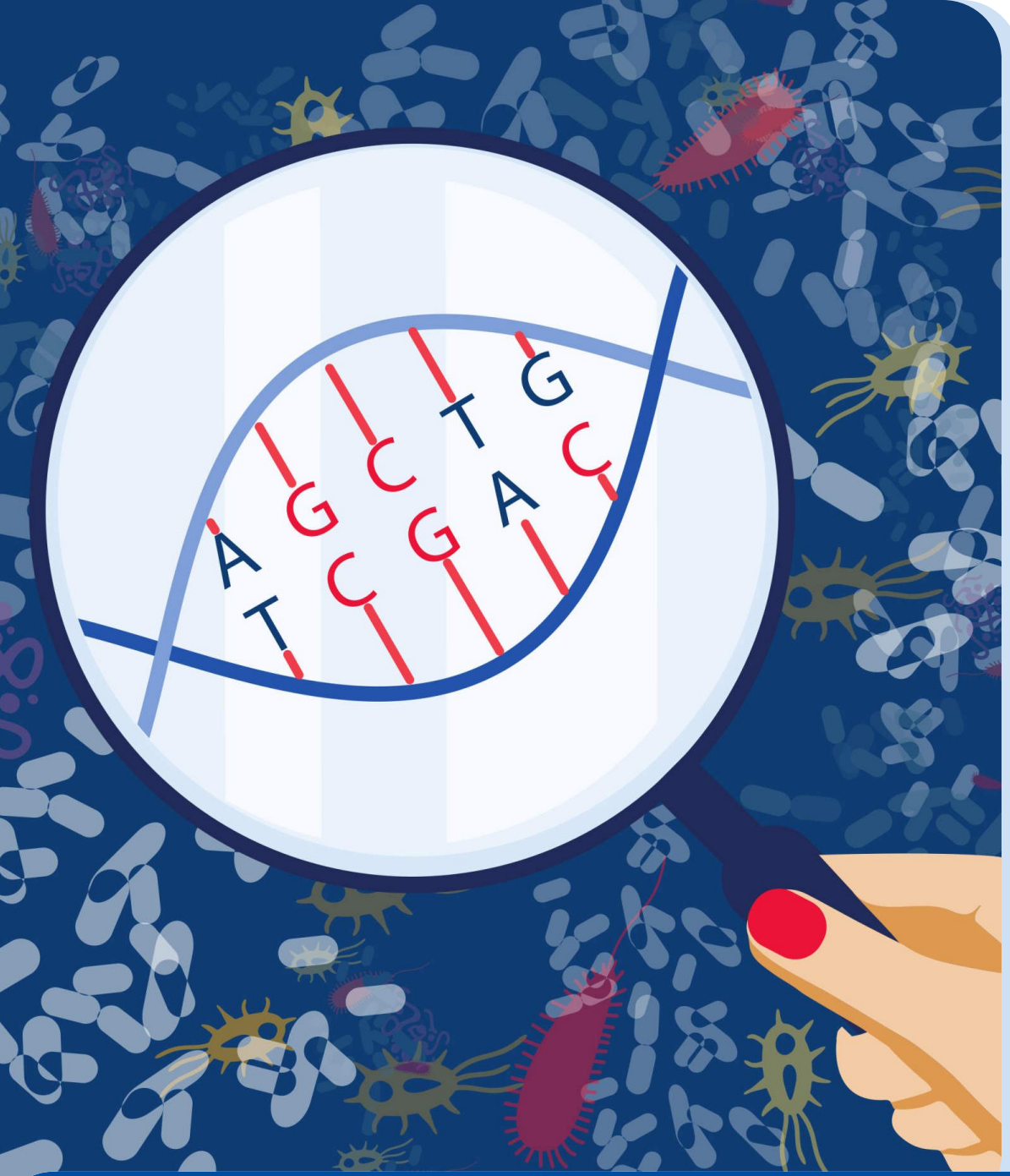
Final thoughts

- **Always consider the whole workflow – not just the reagents, but the sampling environment, the processing environment, the operators, etc.**
  - Reagents can be cleaned, but the other factors may be less controllable
  - But do use ultraclean reagents when dealing with low-biomass samples!
- **Run many negative controls!**
  - Try to sequence the negatives even when using ultraclean reagents

Food for thought

- **MASTER project resulted in many requests for the modified UCP PowerSoil Pro kit**
  - We will release this version of the kit early next year
- **Other projects can result in similar new products**



Questions?

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