How important is sample collection and DNA/RNA extraction when profiling microbial communities

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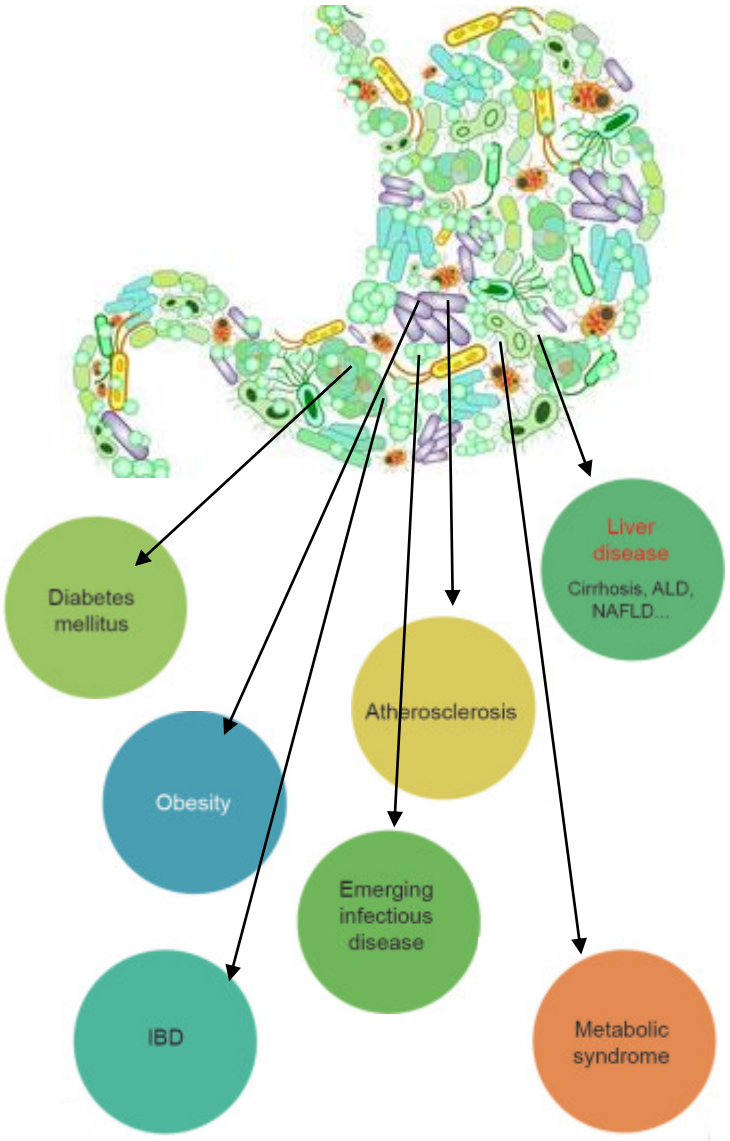
SPES BONA

DRAKENSTEIN CHILD LUNG HEALTH STUDY

The GIT microbiome as example

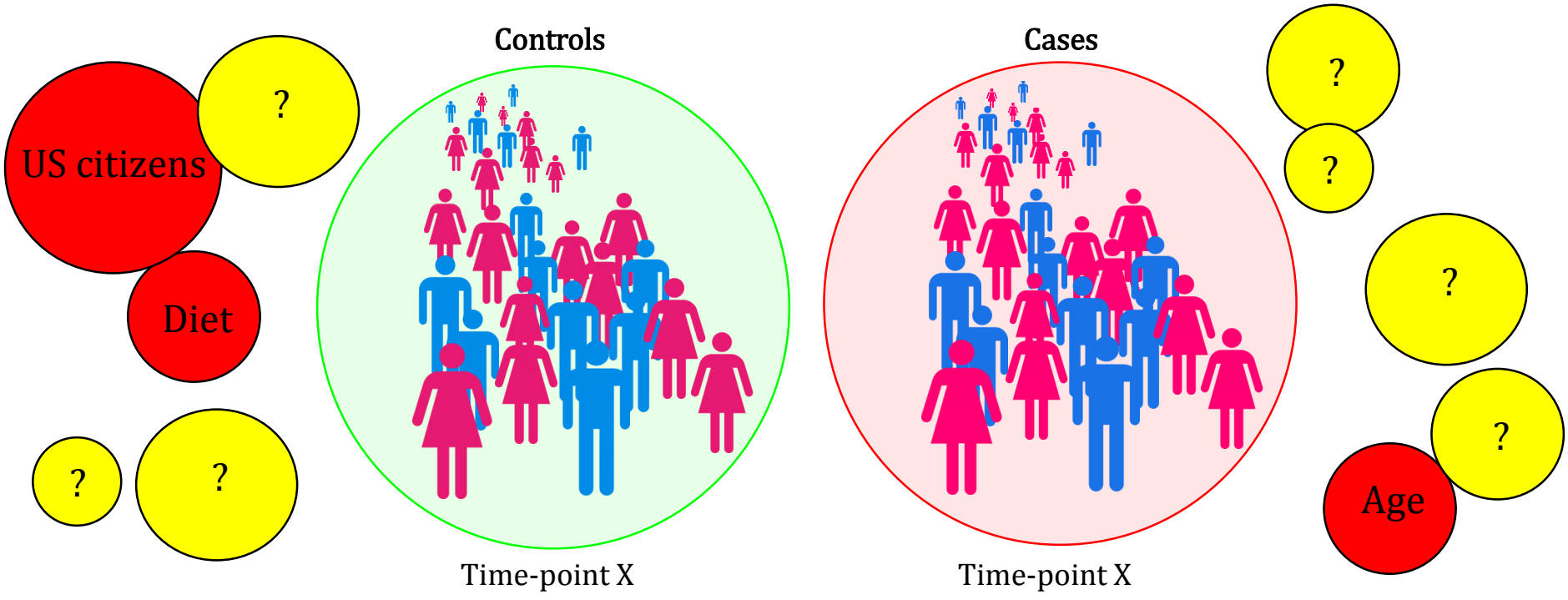
- In recent years, the human gut microbiota has emerged as a primary target area for health monitoring and modulation.
- Alterations in the gut microbiota have been linked repeatedly to pathological states such as infections, autoimmune disorders, inflammatory bowel diseases and cancer.
- Aside from these pathologies, accumulating evidence suggests that gut microbiota composition can serve as indicator of **chronic suboptimal health** and **well-being** either directly linked to suboptimal bowel functioning (e.g. bloating, flatulation, constipation) or extended to general health (e.g. chronic undefined inflammation, anxiety and stress).



Vandeputte et al. (2017) *FEMS Microbiology Reviews* 41:S154–S167; Wang et al. (2017) *Engineering*. 3(1): 71-82

Study design in microbiome studies

- The first major insights on disease-associated microbiome variation have been gained from targeted, medium-sized (N < 400), **cross-sectional** studies.
- Many of these early studies collected only **limited additional data on the study subjects** (e.g. food habits, clinical parameters) and were often **single centre-based or restricted to certain populations** (e.g. US or Chinese citizens).
- However, to effectively tackle health monitoring and modulation through the gut microbiota, substantial targeted research efforts are required.



Vandeputte et al. (2017) *FEMS Microbiology Reviews* 41:S154–S167

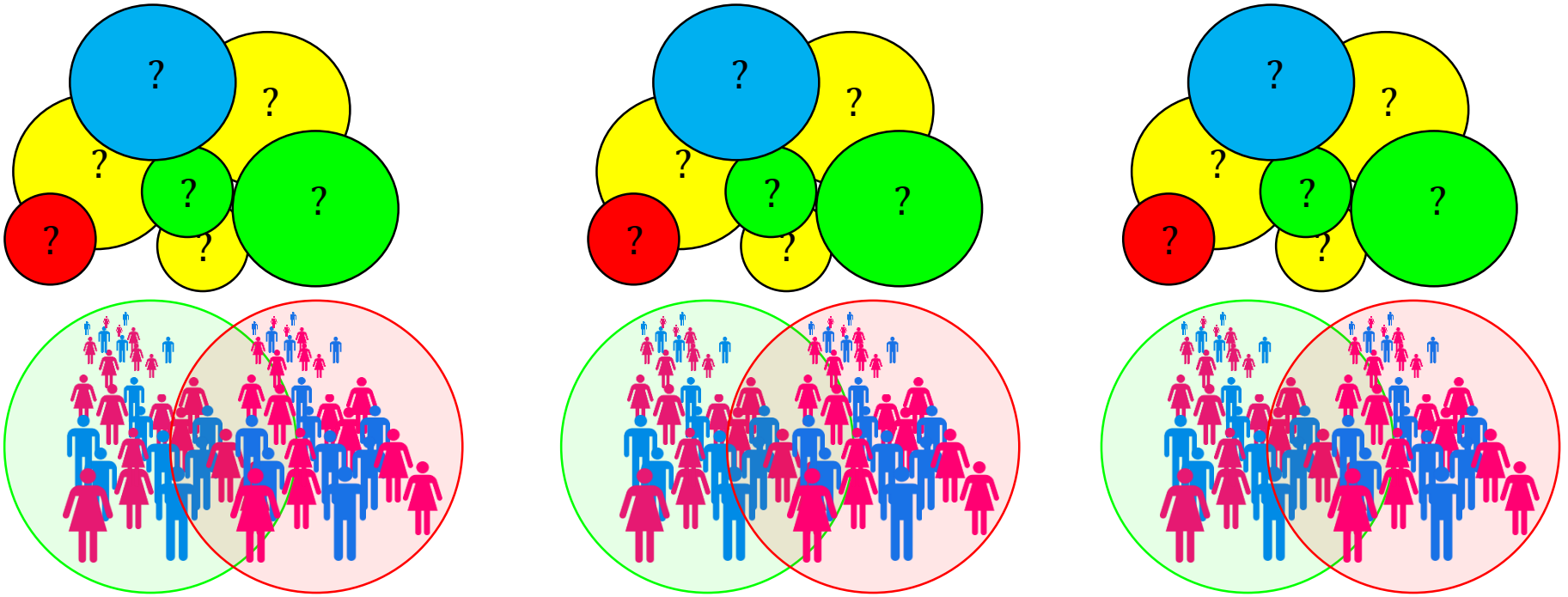
Study design in microbiome studies

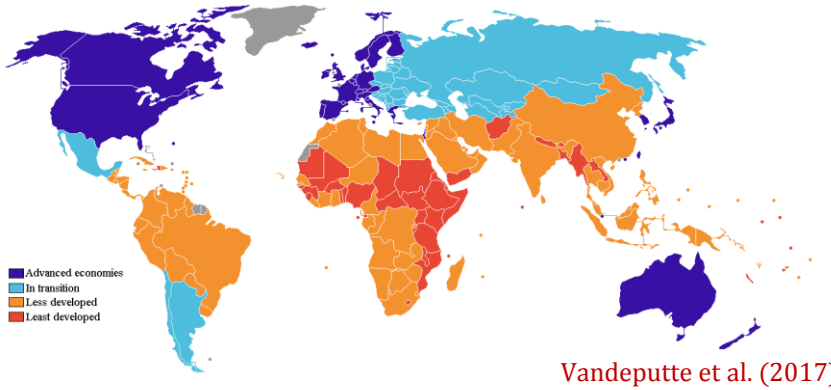
Important considerations:

- Larger, representative population cohorts need to be screened in order to pick up relevant microbiome signals beyond multiple expected confounding factors.
- Many parameters have already been reported to influence gut microbiota composition, ranging from host genotype, nutrition, inflammation and antibiotic usage to stool consistency.
- It has become obvious that upscaling is required to disentangle the multiple, confounding effects in the high-dimensional microbiome.
- Only a longitudinal study design allows encompassing the dynamic nature of these factors and the identification of microbiome-based prognostic signals and markers.
- To further study the temporal stability of the gut microbiota, it is also crucial to collect samples over time.
- The historical lack of sufficiently powered, comprehensively phenotyped, longitudinal studies leads to the baffling observation that it is still unclear what defines a dysbiotic gut microbiota.

Vandeputte et al. (2017) *FEMS Microbiology Reviews* 41:S154–S167

Study design in microbiome studies





Vandeputte et al. (2017) *FEMS Microbiology Reviews* 41:S154–S167

Sample collection strategies in microbiome studies

- Ideally, faecal material intended for microbiome monitoring needs to be **frozen immediately** after sampling in order to **stop the growth of residing bacteria and potential contaminants** and to conserve baseline microbial abundances.

- Subsequently, samples should be stored at –80◦C until DNA extraction.
- As sampling is often performed in the comfort of the participants' home, the latter could cause a significant logistical burden.

- Furthermore, faecal microbiome monitoring efforts risk to suffer from selection biases and drop-out associated to personal aversion towards faecal sampling—especially when sampling procedures are experienced as overly laborious.

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Sample collection strategies in microbiome studies

- The first consortia that sequenced the gut microbiota on a large scale opted for freezing whole faecal samples as soon as possible at –80◦C (either after storage in participants' home freezers (MetaHIT)) or in an isolated box with cooled gel packs for a max of 24 h (HMP).

- Since then a multitude of alternative sampling and storage methods have been developed in order to increase user experience or to allow more flexible transport schemes.

- Instead of immediate freezing, samples are stored at 4◦C or RT for several hours, days or even weeks, with or without stabilisation buffer.

- The next slide summarises and discusses a selection of some of today's most popular alternatives, each with their respective practical **advantages** and **disadvantage**.

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Sample collection strategies in microbiome studies

Table 1. Overview of applicable omics techniques, advantages and disadvantages, and a quality assessment of the observed microbiome composition of currently used or tested sample preservation options based on our interpretation of the significant effect of different storage methods from Table 2, with the advised storage period.

	Metagenomics	Transcriptomics	Metabolomics	Advantages	Disadvantages	Quality observed microbiome composition*
Freezing (−20°C)	✓	✓	✓	Culturing possible Long-term storage	Need for equipment and electricity Cold chain management necessary	*****
No buffer (4°C)	<24 [a,b]	✗	<2h[c]	Slows down bacterial growth Slows down fermentation Culturing possible	Need for equipment and electricity Limited amount of time (hours)	*****
No buffer (RT)	<24h[a,d]	✗	✗	No need for equipment and electricity Affordable (e.g. dry swabs, tubes)	Limited amount of time (hours) With swabs: initial weight not determinable	*
TE buffer (RT)	<24h[b]	✗	✗	No need for equipment and electricity	Quantitative analyses need upfront weighing step Limited amount of time (days)	*****
RNA later (RT)	<24h[e,f]	<6d[g]	✗	No need for equipment and electricity	Lower DNA yield[e,h] Lower DNA purity[f] Expensive (relative compared to freezing) Quantitative analyses need upfront weighing step Limited amount of time (days)	**
Ethanol (95%) (RT)	<2d[i]	<2d[i]	<4d[i]	No need for equipment and electricity	Lower DNA yield[k] Flammable reagent (increased shipping costs) Removal of buffer necessary Quantitative analyses need upfront weighing step Limited amount of time (days)	*****
RTTVs (RT)	✓	<28d[i]	✗	No need for equipment and electricity High DNA and RNA recovery[l] Immediate homogenization	Quantitative analyses need upfront weighing step Limited amount of time (weeks) Expensive (relative compared to freezing)	***
Carry Blair (RT)	✓	✓	✓	No need for equipment and electricity Affordable Culturing possible	Overgrowth gram negative bacteria likely Limited amount of time (hours-days) With swabs: lower DNA yield and initial weight not determinable	*****
FTA cards (RT)	✓	✗	✗	No need for equipment and electricity	Initial weight not determinable Small amount of sample, not easy to process	**
FOBT/FIT (RT)	✓	✗	✗	No need for equipment and electricity Large sample collections available from colon cancer screenings	Initial weight not determinable Small amount of sample	*****

*Assessment based on the information from Table 2.

[a]Tedjo *et al.* (2015); [b]Choo, Leong and Rogers (2015); [c]Gratton *et al.* (2016); [d]Carroll *et al.* (2012); [e]Gorzelak *et al.* (2015); [f]Dominianni *et al.* (2014); [g]Reck *et al.* (2015); [h]Hale *et al.* (2015); [i]Franzosa *et al.* (2014); [j]Loftfield *et al.* (2016); [k]Song *et al.* (2016); [l]Anderson *et al.* (2016).

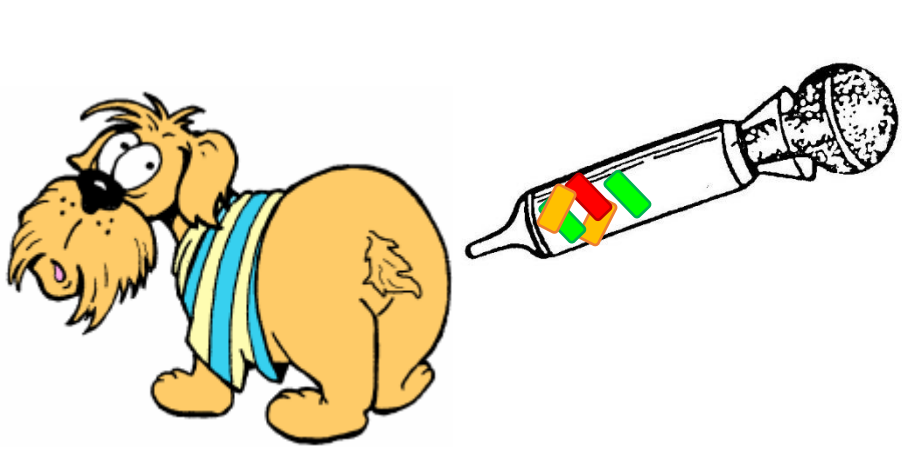
Vandeputte et al. (2017) *FEMS Microbiology Reviews* 41:S154–S167

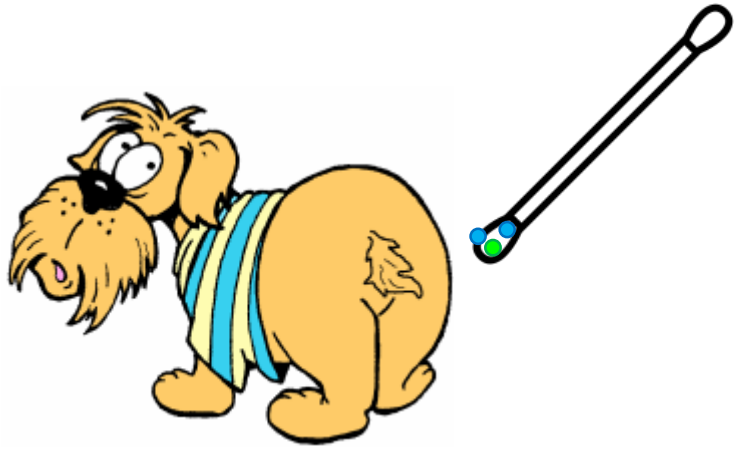
Sample collection strategies in microbiome studies

- Whichever method is chosen, it is crucial that samples are all treated the same.
- Buffers added to preserve specimens may contain a “microbiota composition” of their own which will result in additional background added to specimens. If case specimens are for example stored without the buffer, whilst controls are stored with, false difference will be detected between the two groups under study.

Sample collection strategies in microbiome studies

- Sample collection also need to be performed in the exact same manner for all samples under study., for example:
  - If stool samples are collected via aspiration, microbiota background may be introduced to the stool specimens via the solution used to aspirate the sample. Caution should therefore be taken when comparing microbiota profiles from aspirated samples and those collected during passing of stool.
  - If a swab is used to collect the specimen, the same supplier and product should be used throughout the project.





Nucleic acid extraction

- A wide range of DNA extraction kits are available on the market, combining premade buffers, materials, and protocols for the disruption of cellular membranes, denaturation of proteins, and purification of nucleic acids thus ensuring reproducibility and reliability.

- Many of these commercial methods contain similar components such as guanidine-based chaotropic salts and silica-adsorption spin-columns, but **kits might vary** in the **composition of the buffers and enzymes used for cell lysis** and whether **mechanical lysis steps such as bead-beating are incorporated** or not.

Bik (2016) *Yale Journal Of Biology And Medicine*. 89: 363-373

Nucleic acid extraction

- The QIAamp (Qiagen, Valencia, CA) and PowerSoil (MO BIO Laboratories, Carlsbad, CA) DNA extraction kits are currently among the most popular choices for microbiome analysis applications.

Bik (2016) *Yale Journal Of Biology And Medicine*. 89: 363-373

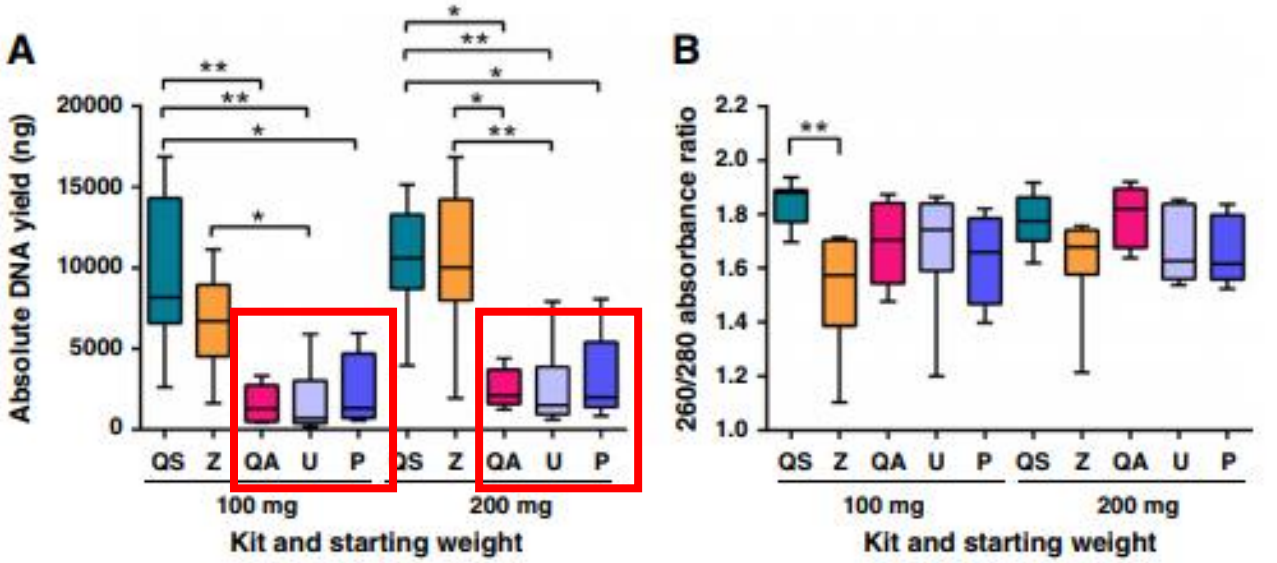
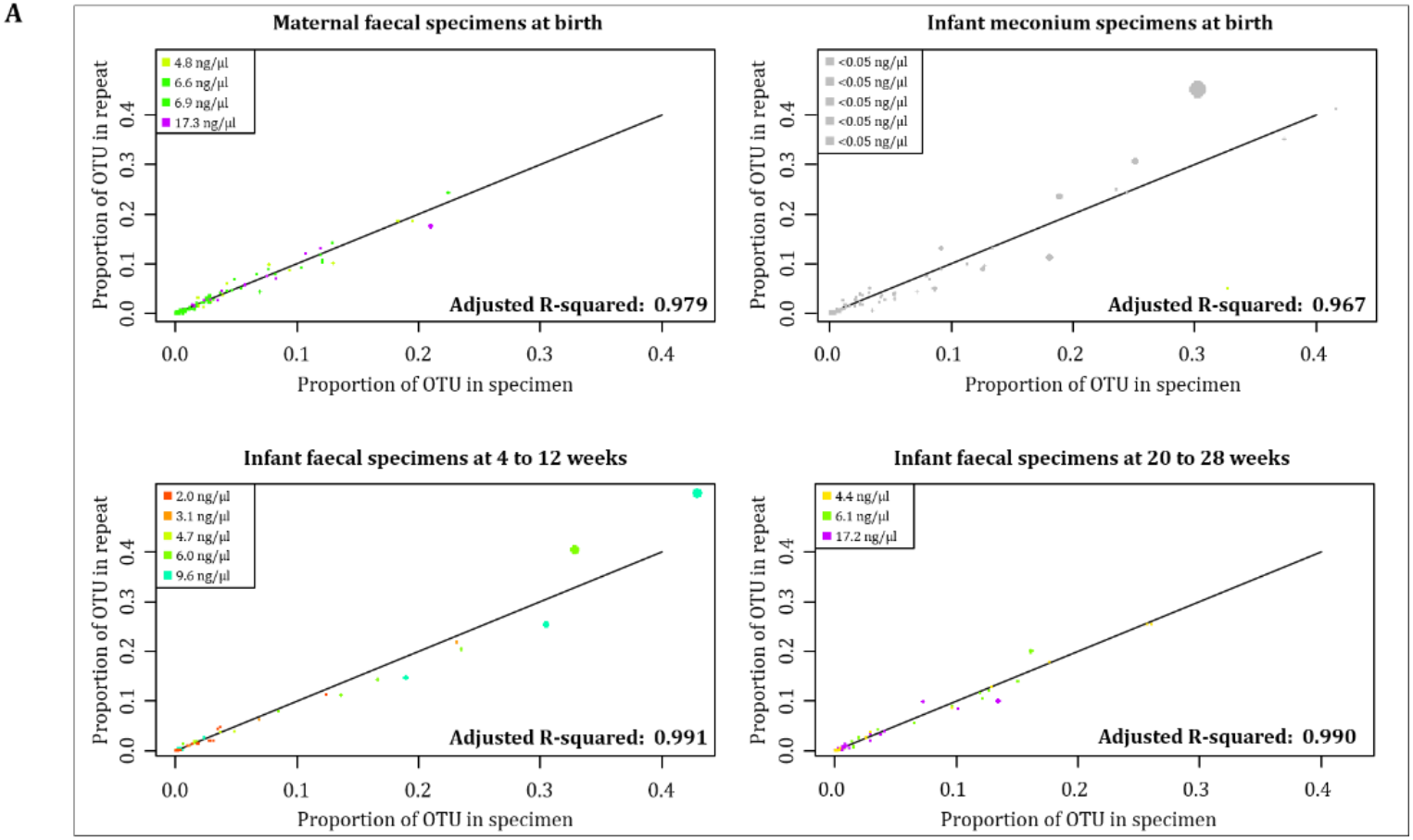


Fig. 1. DNA yield and purity extracted from faecal specimens using five commercial kits. DNA was extracted in triplicate from two starting weights (100 mg and 200 mg) of faeces per sample (n = 8 samples per kit). Median values are indicated by the line within the box plot. The box extends from the 25th to 75th percentiles and whiskers indicate the minimum and maximum values. * $p < 0.05$; ** $p \leq 0.01$. A) Boxplot showing DNA yields obtained for all five kits evaluated. Kit QA values after RNA degradation and clean-up. B) Figure showing absorbance ratios at 260/280 for all five kits assessed. Kit QA values after RNA degradation. QS: QIAsymphony® Virus/Bacteria Mini Kit (Qiagen); Z: ZR Fecal DNA Isolation Kit™ (Zymo Research); QA: QIAamp® DNA Stool Mini Kit (Qiagen); U: Ultraclean® Fecal DNA Isolation Kit (Mobio); P: PowerSoil® DNA Isolation Kit (Mobio).

Claassen et al. (2013) *Journal of Microbiological Methods* 94:103–110

Nucleic acid extraction

- Although it has been reported that the number of reads sequenced influences reproducibility, recent analysis performed by our group actually showed that **nucleic acid concentration** seem to have an even larger effect:



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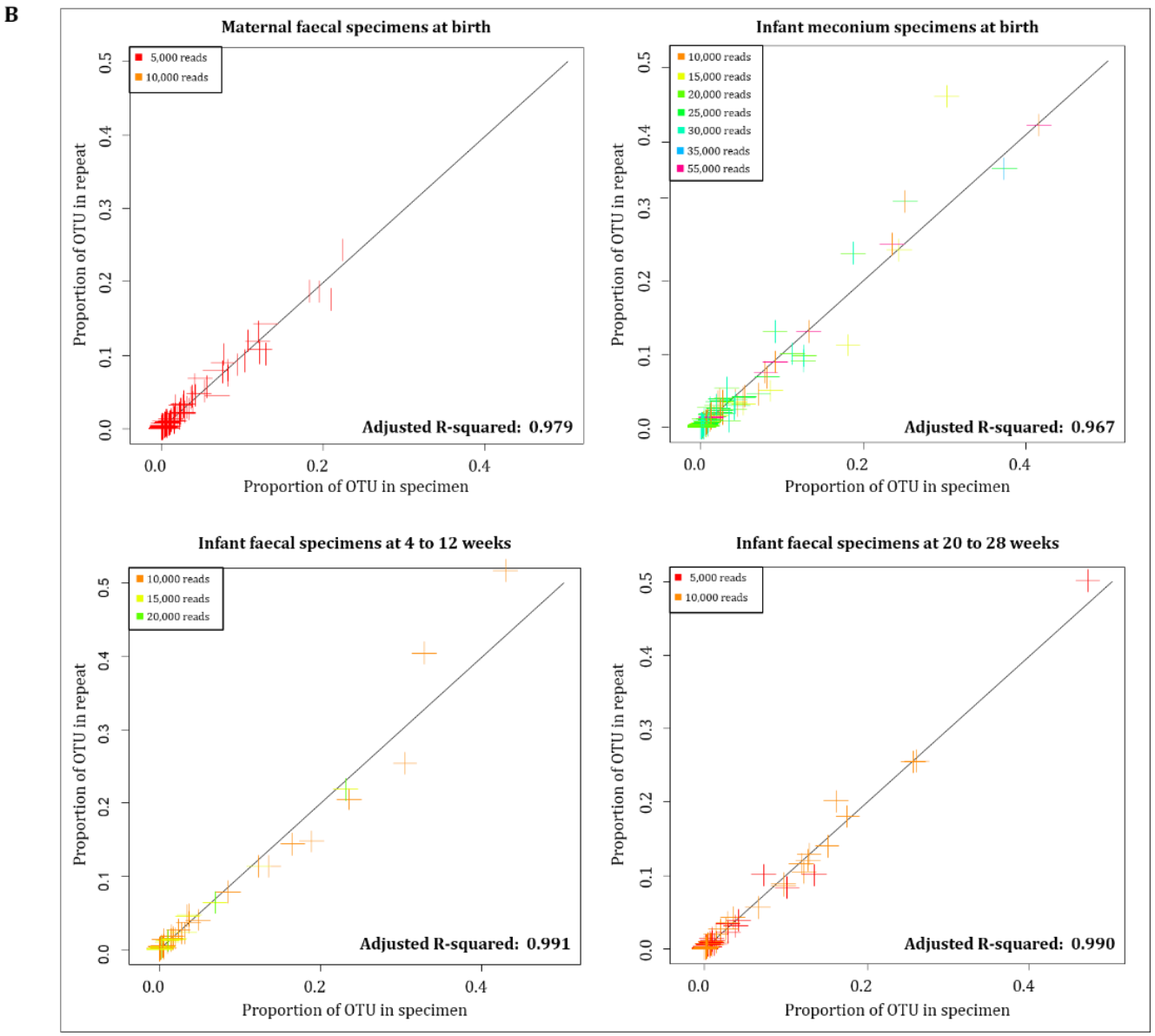
Nucleic acid extraction



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Nucleic acid extraction

- Depending on the type of specimen to be extracted, the choice of nucleic acid extraction kit might have a considerable effect on both the yield as well as the bacterial ratios in the purified sample.

Evaluating variation in human gut microbiota profiles due to DNA extraction method and inter-subject differences

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The human gut contains dense and diverse microbial communities which have profound influences on human health. Gaining meaningful insights into these communities requires provision of high quality microbial nucleic acids from human fecal samples, as well as an understanding of the sources of variation and their impacts on the experimental model. We present here a systematic analysis of commonly used microbial DNA extraction methods, and identify significant sources of variation. Five extraction methods (Human Microbiome Project protocol, MoBio PowerSoil DNA Isolation Kit, QIAamp DNA Stool Mini Kit, ZR Fecal DNA MiniPrep, phenol:chloroform-based DNA isolation) were evaluated based on the following criteria: DNA yield, quality and integrity, and microbial community structure based on Illumina amplicon sequencing of the V4 region of bacterial and archaeal 16S rRNA genes. Our results indicate that the largest portion of variation within the model was attributed to differences between subjects (biological variation), with a smaller proportion of variation associated with DNA extraction method (technical variation) and intra-subject variation. A comprehensive understanding of the potential impact of technical variation on the human gut microbiota will help limit preventable bias, enabling more accurate diversity estimates.

Contamination

- An important point to address is the possible introduction of contaminating DNA during sample preparation.

- Contamination might occur during several stages of the sample processing by cross-contamination from adjacent samples, the operator, or the presence of amplicon residues in the laboratory. Important measures to reduce these types of contamination are the use of biosafety cabinets, gloves, filter tips, and separate areas for DNA extraction and PCR.

- In addition, several studies have reported the presence of low amounts of contaminant DNA in sample collection materials such as paper points used for the collection of oral samples, DNA extraction buffers or columns, or PCR reagents, a problem that is much harder to avoid.

Bik (2016) *Yale Journal Of Biology And Medicine*. 89: 363-373

Contamination

- The concern for contamination becomes increasingly important when extracting or amplifying low-yield clinical samples, such as blood, where the signal-to-noise ratio is low.

- In an elegant study, Salter et al. showed that most DNA extraction reagents contain non-negligible amounts of contaminating DNA that could progressively be more detected in samples with a low amount of microbial biomass.

- An increasing number of studies attempt to detect microbial DNA in near-sterile environments such as amniotic fluid or blood from healthy individuals. Without the inclusion of carefully selected extraction and amplification controls, the interpretation of the results of such studies becomes very difficult.

Bik (2016) *Yale Journal Of Biology And Medicine*. 89: 363-373

Which 16S rRNA hypervariable region to target

- Due to its ubiquity in prokaryotes, low horizontal gene transfer, and ability to differentiate closely related organisms, the 16S rRNA gene has been used for decades in the study of diversity and ecology of microorganisms.

- However, most NGS platforms are not capable of covering the full length of the gene (ca. 1,500 bp).

- This is why short regions within the gene (e.g., hypervariable V1–V9 regions) have been prioritized with the advent of these newer technologies.

- Hypervariable regions are supposed to act as proxies of the complete gene. Actually, there is correlation between the phylogenies generated using different hypervariable regions or combinations thereof and the phylogenies generated with the whole gene, but the strength of these correlations varies among regions because their different evolutionary rates limit their capacity to serve as surrogates of full-length sequences.

De la Cuesta-Zuluaga (2016) *Frontiers in Nutrition*. 3: Article 26

Which 16S rRNA hypervariable region to target

- Because of these disparities, the OTU count of different 16S regions can be inconsistent, which, in turn, makes studies using different hypervariable regions incomparable.

- Currently, there is no consensus of which region best reflects the gut microbial community.

- While read length increases in newer NGS technologies, one empirical way to overcome comparability between studies would be to sequence the same hypervariable region. This is, indeed, what is seen in many gut microbiome studies today: since the Illumina MiSeq platform gives one of the bests value for money of all NGS, most microbiome researchers are moving to sequence the V4 region since its size (ca. 250 bp) fits well the read size of this platform at its current version.

De la Cuesta-Zuluaga (2016) *Frontiers in Nutrition*. 3: Article 26

Which 16S rRNA hypervariable region to target

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PLoS one

Target Region Selection Is a Critical Determinant of Community Fingerprints Generated by 16S Pyrosequencing

Purnima S. Kumar[1]*, Michael R. Brooker[1], Scot E. Dowd[2], Terry Camerlengo[3]

1 Division of Periodontology, College of Dentistry, The Ohio State University, Columbus, Ohio, United States of America, 2 Research Testing Laboratory, Lubbock, Texas, United States of America, 3 Comprehensive Cancer Center, The Ohio State University, Columbus, Ohio, United States of America

Abstract

Pyrosequencing of 16S rRNA genes allows for in-depth characterization of complex microbial communities. Although it is known that primer selection can influence the profile of a community generated by sequencing, the extent and severity of this bias on deep-sequencing methodologies is not well elucidated. We tested the hypothesis that the hypervariable region targeted for sequencing and primer degeneracy play important roles in influencing the composition of 16S pyrotag communities. Subgingival plaque from deep sites of current smokers with chronic periodontitis was analyzed using Sanger sequencing and pyrosequencing using 4 primer pairs. Greater numbers of species were detected by pyrosequencing than by Sanger sequencing. Rare taxa constituted nearly 6% of each pyrotag community and less than 1% of the Sanger sequencing community. However, the different target regions selected for pyrosequencing did not demonstrate a significant difference in the number of rare and abundant taxa detected. The genera *Prevotella*, *Fusobacterium*, *Streptococcus*, *Granulicatella*, *Bacteroides*, *Porphyromonas* and *Treponema* were abundant when the V1–V3 region was targeted, while *Streptococcus*, *Treponema*, *Prevotella*, *Eubacterium*, *Porphyromonas*, *Campylobacer* and *Enterococcus* predominated in the community generated by V4–V6 primers, and the most numerous genera in the V7–V9 community were *Veillonella*, *Streptococcus*, *Eubacterium*, *Enterococcus*, *Treponema*, *Catonella* and *Selenomonas*. Targeting the V4–V6 region failed to detect the genus *Fusobacterium*, while the taxa *Selenomonas*, *TM7* and *Mycoplasma* were not detected by the V7–V9 primer pairs. The communities generated by degenerate and non-degenerate primers did not demonstrate significant differences. Averaging the community fingerprints generated by V1–V3 and V7–V9 primers providesd results similar to Sanger sequencing, while allowing a significantly greater depth of coverage than is possible with Sanger sequencing. It is therefore important to use primers targeted to these two regions of the 16S rRNA gene in all deep-sequencing efforts to obtain representational characterization of complex microbial communities.

Which 16S rRNA hypervariable region to target

Skin Microbiome Surveys Are Strongly Influenced by Experimental Design

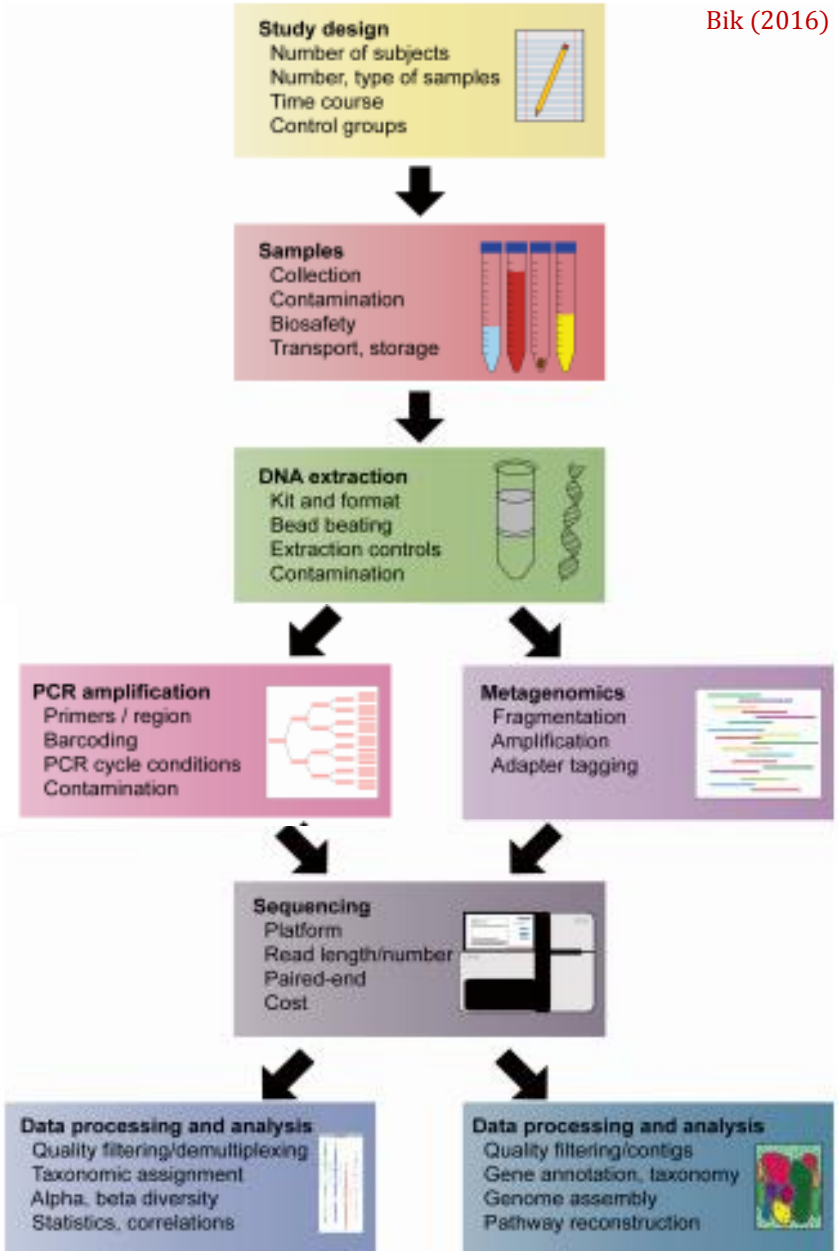
Jacquelyn S. Meisel[1], Geoffrey D. Hannigan[1], Amanda S. Tyldsley[1], Adam J. SanMiguel[1], Brendan P. Hodkinson[1], Qi Zheng[1] and Elizabeth A. Grice[1]

Culture-independent studies to characterize skin microbiota are increasingly common, due in part to affordable and accessible sequencing and analysis platforms. Compared to culture-based techniques, DNA sequencing of the bacterial 16S ribosomal RNA (rRNA) gene or whole metagenome shotgun (WMS) sequencing provides more precise microbial community characterizations. Most widely used protocols were developed to characterize microbiota of other habitats (i.e., gastrointestinal) and have not been systematically compared for their utility in skin microbiome surveys. Here we establish a resource for the cutaneous research community to guide experimental design in characterizing skin microbiota. We compare two widely sequenced regions of the 16S rRNA gene to WMS sequencing for recapitulating skin microbiome community composition, diversity, and genetic functional enrichment. We show that WMS sequencing most accurately recapitulates microbial communities, but sequencing of hypervariable regions 1–3 of the 16S rRNA gene provides highly similar results. Sequencing of hypervariable region 4 poorly captures skin commensal microbiota, especially *Propionibacterium*. WMS sequencing, which is resource and cost intensive, provides evidence of a community's functional potential; however, metagenome predictions based on 16S rRNA sequence tags closely approximate WMS genetic functional profiles. This study highlights the importance of experimental design for downstream results in skin microbiome surveys.

Journal of Investigative Dermatology (2016) **136,** 947–956; doi:10.1016/j.jid.2016.01.016

Primary steps to consider when conducting microbiota studies

Bik (2016) *Yale Journal Of Biology And Medicine*. 89: 363-373



In summary

Study design needs careful thought

Use optimized sampling procedures for the sample type collected

Nucleic acid extraction procedures determine microbiota profiles and impact on sequencing reproducibility

Include optimal controls for assessing and correcting contamination

Different hypervariable regions of the 16S rRNA gene are targeted when analyzing different sample types

Other important factors to consider: the sequencing process itself and data processing and analysis