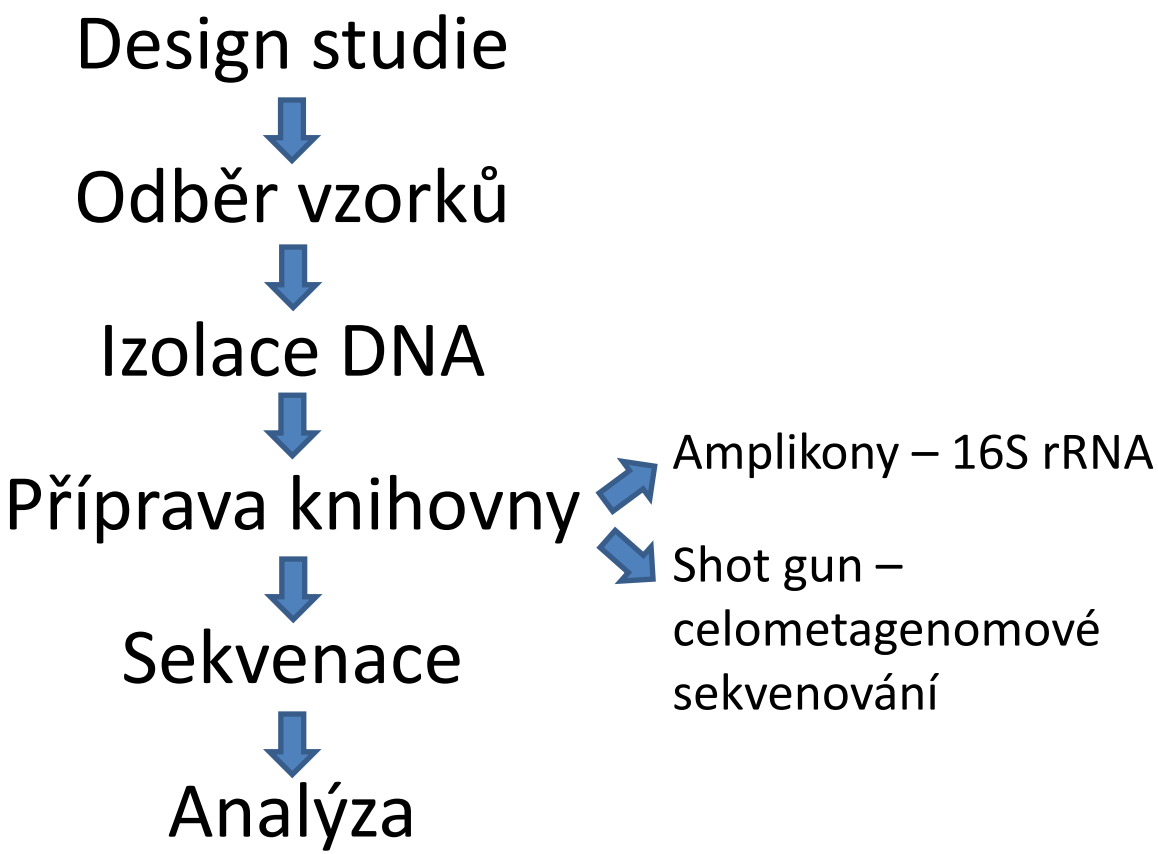
Příprava vzorků – odběr vzorků, uchování vzorků a izolace

Petra Vídeňská, Ph.D.

Workflow


Design studie
Odběr vzorků
Izolace DNA
Příprava knihovny
Amplikony – 16S rRNA
Shot gun – celometagenomové sekvenování
Sekvenace
Analýza

Nejčastější typy vzorků

- Voda
- Půda
- Biologické vzorky
  - Stolice
  - Sliny
  - Stěry

!Nejdůležitější je vždy zachovat stejný postup odběru!

Odběr vzorků - půda

- Vhodně zvolit lokalitu, možno i z několika hloubek a vícero opakování vzorkování
- Oděr do sterilní nádoby, sáčku
- Transport na ledu, následné zmražení na -20 °C až -80°C
- Na izolaci většinou postačí malé množství půdy, často se ale měří vícero hodnot a odebírá se tedy velké množství – půdu je před zpracováním nutno homogenizovat

Odběr vzorků - voda

- Možno odebírat z více hloubek ve zvolené lokalitě
- Odebírá se větší objem (dle očekáváné bakteriální kontaminace 100 – 1000 ml) a transportuje se na ledu do laboratoře
- Následně se voda filtruje přes bakteriologický filtr
- Filtr je vložen do vhodné tekutiny (voda, TE, PBS) a promyje se

Odběr vzorků – biologické vzorky

- složitější, např. u stolice velké množství anaerobů – fakultativně anaerobní bakterie rychle přerůstají
- Nutno okamžitě zamrazit
- Pokud není možno – komerční kity s pufry sloužící ke stabilizaci DNA a zamezení přerůstání bakterií po dobu 14 dnů

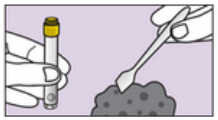
Komerční souprava na odběr vzorků stolice

OMNIgene•GUT (OMR-200)

1

Unscrew the purple cap from the collection device and set it aside for later use.
IMPORTANT:
Do NOT remove the yellow tube top.
Do NOT spill the stabilizing liquid in the tube.

2



Use the stick to collect a small amount of fecal sample.

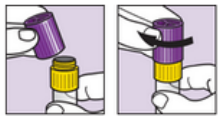
3

Transfer the fecal sample into the yellow tube top
Repeat until the sample reaches the top and fills it completely.
IMPORTANT: Do NOT push sample into the tube.

4

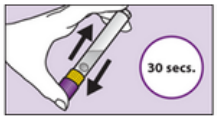
Scrape horizontally across the tube top to level the sample and remove any excess. Discard the stick. Wipe exterior of tube and top with toilet paper or tissue as needed.

5



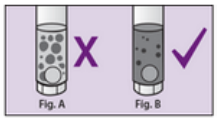
Screw the purple cap back onto the yellow tubetop until tightly closed.

6



Shake the sealed tube as hard and fast as possible in a back and forth motion for a minimum of 30 seconds.

7



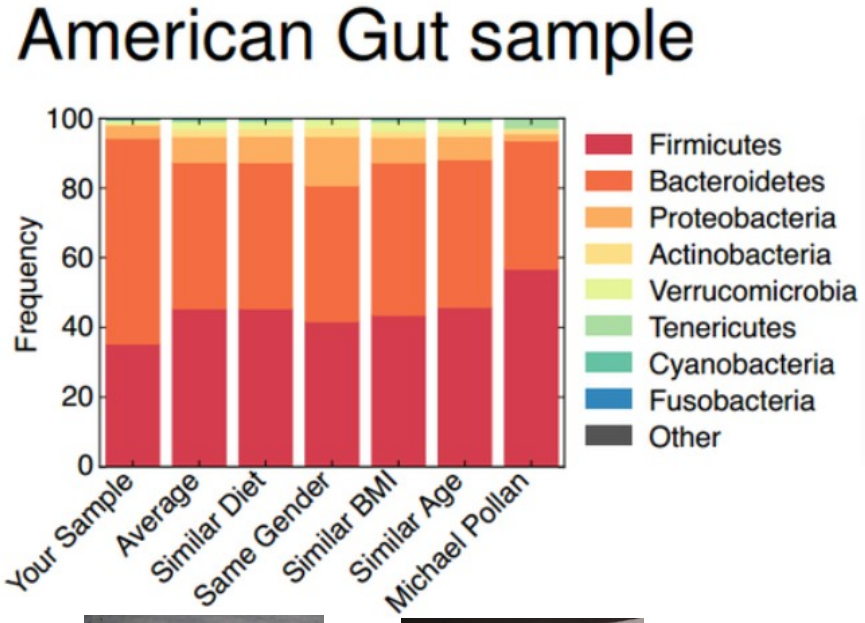
The fecal sample will be mixed with the stabilizing liquid in the tube; not all particles will dissolve.
IMPORTANT: Continue shaking if large particles remain as shown in Figure A.
Send the sample for processing following the delivery instructions supplied by the kit provider.

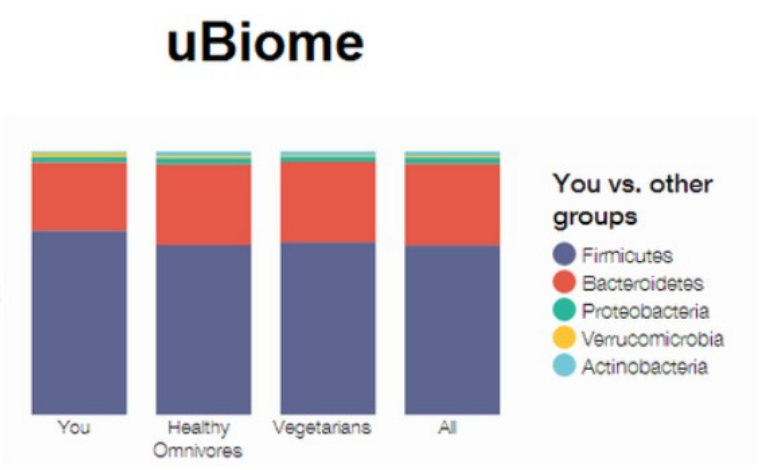
Odběr vzorků stolice - problémy

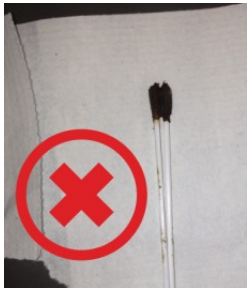
- Nestandardizované odběry vzorků – různá délka vystavení vzorku kyslíku před zamrazením
- Problém homogenizace – odběr jen z jednoho místa stolice
- Přítomnost inhibitorů PCR

Ukázka různých přístupů – American Gut Project x uBiome

- https://mrheisenbug.wordpress.com/2014/04/24/dear-american-gut-ubiome-you-have-some-explaining-to-do/







American Gut Project – podmínky skladování

- http://www.ncbi.nlm.nih.gov/pubmed/20412303

FEMS Microbiol Lett. Author manuscript; available in PMC 2011 August 1.

Published in final edited form as:
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Effect of storage conditions on the assessment of bacterial community structure in soil and human-associated samples

Christian L. Lauber[1,*], **Nicholas Zhou**[2], **Jeffrey I. Gordon**[3], **Rob Knight**[4,5], and **Noah Fierer**[1,2]

[1]Cooperative Institute for Research in Environmental Sciences, University of Colorado, UCB 216, Boulder, CO 80309

[2]Department of Ecology and Evolutionary Biology, University of Colorado, UCB 334, Boulder, CO 80309

[3]Center for Genome Sciences, Washington University School of Medicine, St. Louis, MO 63108

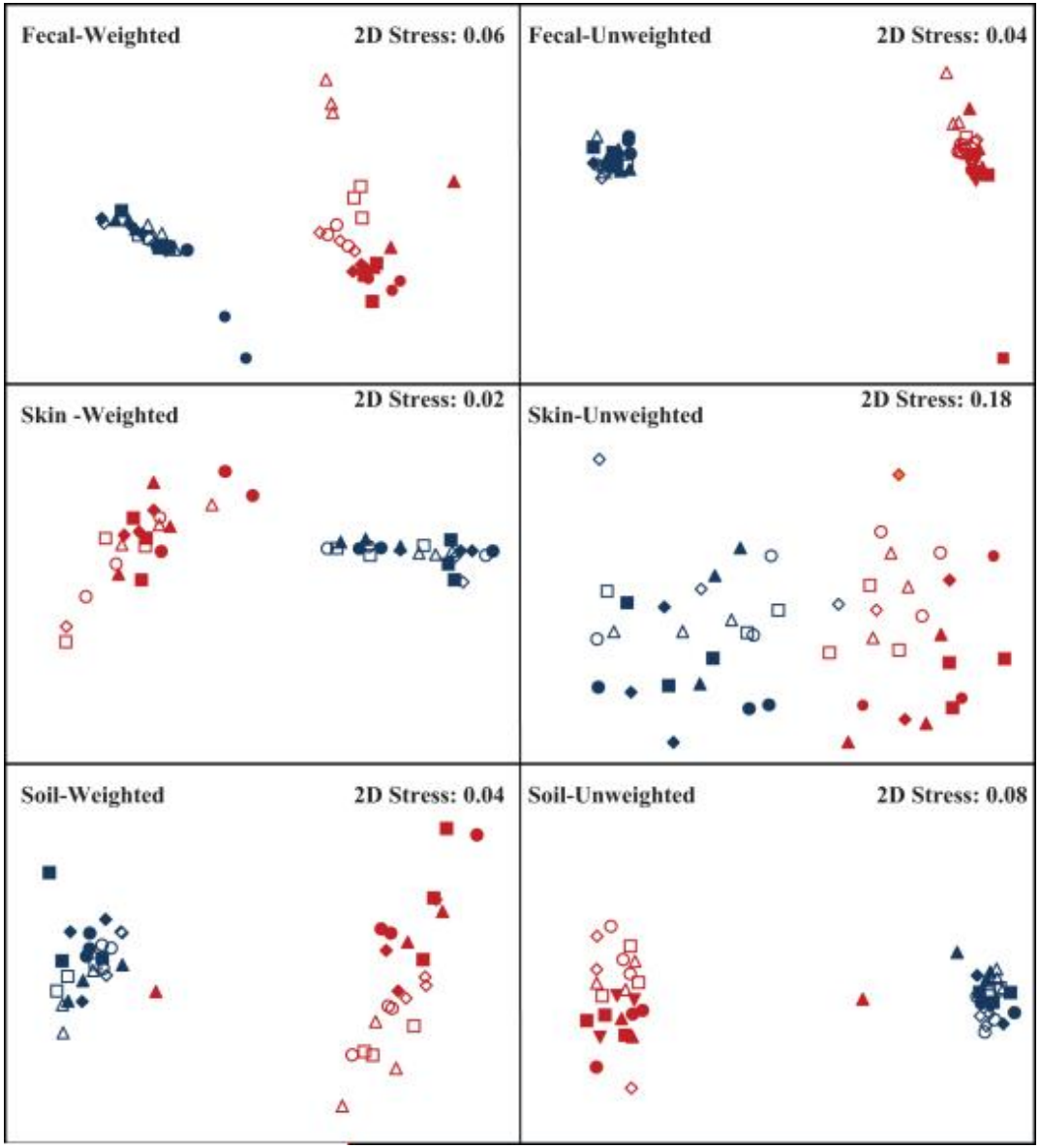
[4]Department of Chemistry and Biochemistry, University of Colorado, UCB 215, Boulder, CO 80309

[5]Howard Hughes Medical Institute

Abstract

Storage conditions are considered to be a critical component of DNA-based microbial community analysis methods. However, whether differences in short-term sample storage conditions impact the assessment of bacterial community composition and diversity demands systematic and quantitative assessment. Therefore, we used barcoded pyrosequencing of bacterial 16S rRNA genes to survey communities, harvested from a variety of habitats (soil, human gut (feces) and human skin) and subsequently stored at 20°, 4°, −20°, and −80°C for 3 and 14 days. Our results indicate that the phylogenetic structure and diversity of communities in individual samples was not significantly influenced by storage temperature or duration of storage. Likewise, the relative abundances of most taxa were largely unaffected by temperature even after 14 days of storage. Our results indicate that environmental factors and biases in molecular techniques likely impart greater amounts of variation to microbial communities than do differences in short-term storage conditions, including storage for up to two weeks at room temperature. These results suggest that many samples collected and stored under field conditions without refrigeration may be useful for microbial community analyses.

American Gut Project – podmínky skladování



Non-metric Multidimensional Scaling (NMDS) plots of UniFrac weighted and unweighted pairwise distances. Overall community composition was not affected by temperature or duration of storage for weighted UniFrac distances ($P > 0.1$ in all cases). Length of storage significantly affected the skin communities for the unweighted UniFrac metric ($P = 0.02$). The remaining unweighted UniFrac distances were not significantly different by day or temperature. Blue=sample 1, red = sample 2. Open symbols = Day 3, closed symbols = Day 14. ▲ = 20°C, ■ = 4°C, ● = −20°C, ◆ = −80°C.

Vliv laboratorní teploty na degradaci DNA

Cardona *et al. BMC Microbiology* 2012, **12**:158
http://www.biomedcentral.com/1471-2180/12/158

BMC Microbiology

RESEARCH ARTICLE Open Access

Storage conditions of intestinal microbiota matter in metagenomic analysis

Silvia Cardona[1], Anat Eck[1], Montserrat Cassellas[1], Milagros Gallart[1], Carmen Alastrue[1], Joel Dore[2], Fernando Azpiroz[1], Joaquim Roca[3], Francisco Guarner[1] and Chaysavanh Manichanh[1*]

Abstract

Background: The structure and function of human gut microbiota is currently inferred from metagenomic and metatranscriptomic analyses. Recovery of intact DNA and RNA is therefore a critical step in these studies. Here, we evaluated how different storage conditions of fecal samples affect the quality of extracted nucleic acids and the stability of their microbial communities.

Results: We assessed the quality of genomic DNA and total RNA by microcapillary electrophoresis and analyzed the bacterial community structure by pyrosequencing the 16S rRNA gene. DNA and RNA started to fragment when samples were kept at room temperature for more than 24 h. The use of RNAse inhibitors diminished RNA degradation but this protection was not consistent among individuals. DNA and RNA degradation also occurred when frozen samples were defrosted for a short period (1 h) before nucleic acid extraction. The same conditions that affected DNA and RNA integrity also altered the relative abundance of most taxa in the bacterial community analysis. In this case, intra-individual variability of microbial diversity was larger than inter-individual one.

Conclusions: Though this preliminary work explored a very limited number of parameters, the results suggest that storage conditions of fecal samples affect the integrity of DNA and RNA and the composition of their microbial community. For optimal preservation, stool samples should be kept at room temperature and brought at the laboratory within 24 h after collection or be stored immediately at −20°C in a home freezer and transported afterwards in a freezer pack to ensure that they do not defrost at any time. Mixing the samples with RNAse inhibitors outside the laboratory is not recommended since proper homogenization of the stool is difficult to monitor.

Keywords: Needs for standardization/RNA and DNA degradation/Metagenomics/16S ribosomal RNA

Vliv laboratorní teploty na degradaci DNA

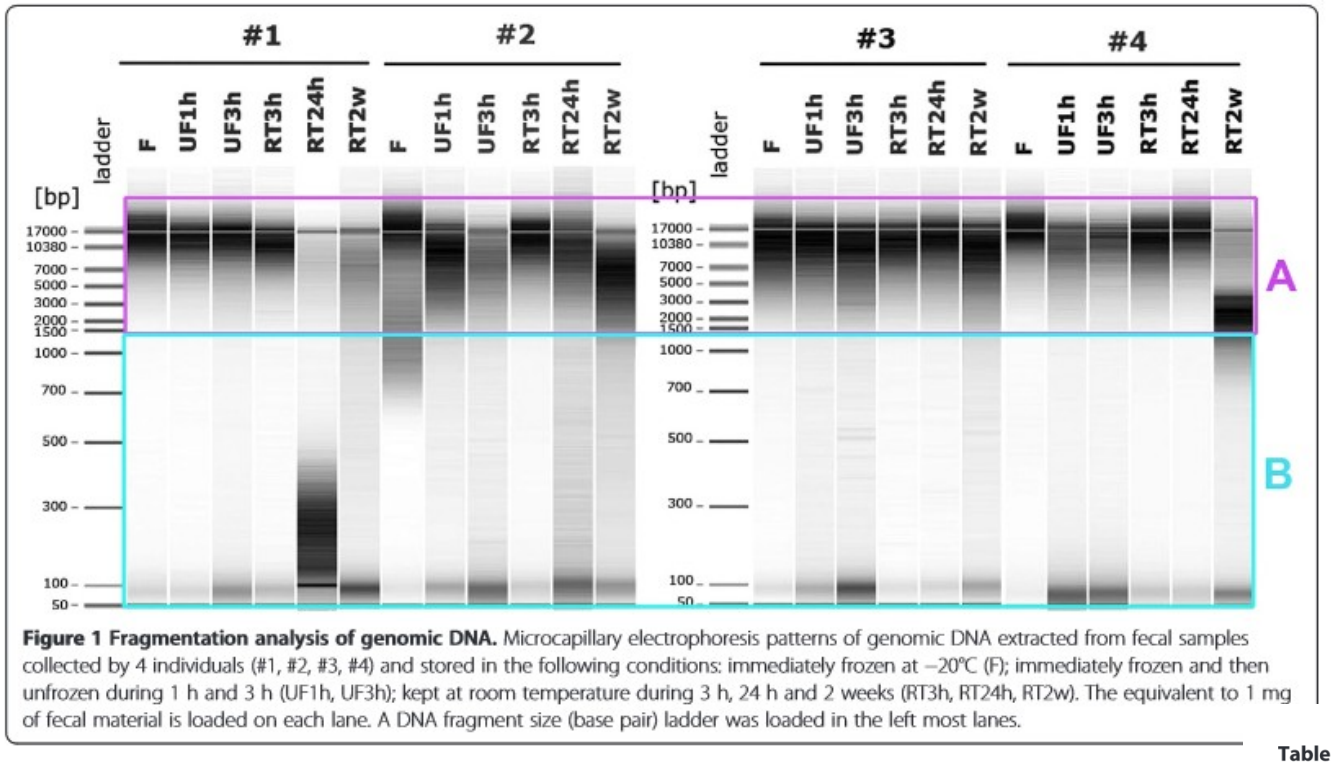


Figure 1 Fragmentation analysis of genomic DNA. Microcapillary electrophoresis patterns of genomic DNA extracted from fecal samples collected by 4 individuals (#1, #2, #3, #4) and stored in the following conditions: immediately frozen at −20°C (F); immediately frozen and then unfrozen during 1 h and 3 h (UF1h, UF3h); kept at room temperature during 3 h, 24 h and 2 weeks (RT3h, RT24h, RT2w). The equivalent to 1 mg of fecal material is loaded on each lane. A DNA fragment size (base pair) ladder was loaded in the left most lanes.

Table 1 Percentage of DNA compared to the frozen samples

	% degraded DNA				n = 4
	#1	#2	#3	#4	*p* value when compared to frozen samples
F	12	28	10	9	
UF1h	12	24	23	34	< 0.01
UF3h	25	39	31	34	< 0.001
RT3h	17	16	12	15	0.9270
RT24h	84	44	13	15	< 0.001
RT2w	48	38	26	40	< 0.001

Statistical analysis was performed using Poisson regression model; *p* value < 0.05 is considered significant; #1, #2, #3, #4 correspond to subjects 1, 2, 3, 4; F = frozen; UF1h = unfrozen during 1 h; UF3h = unfrozen during 3 h; RT = room temperature; 2w = 2 weeks.

Vliv laboratorní teploty na degradaci DNA

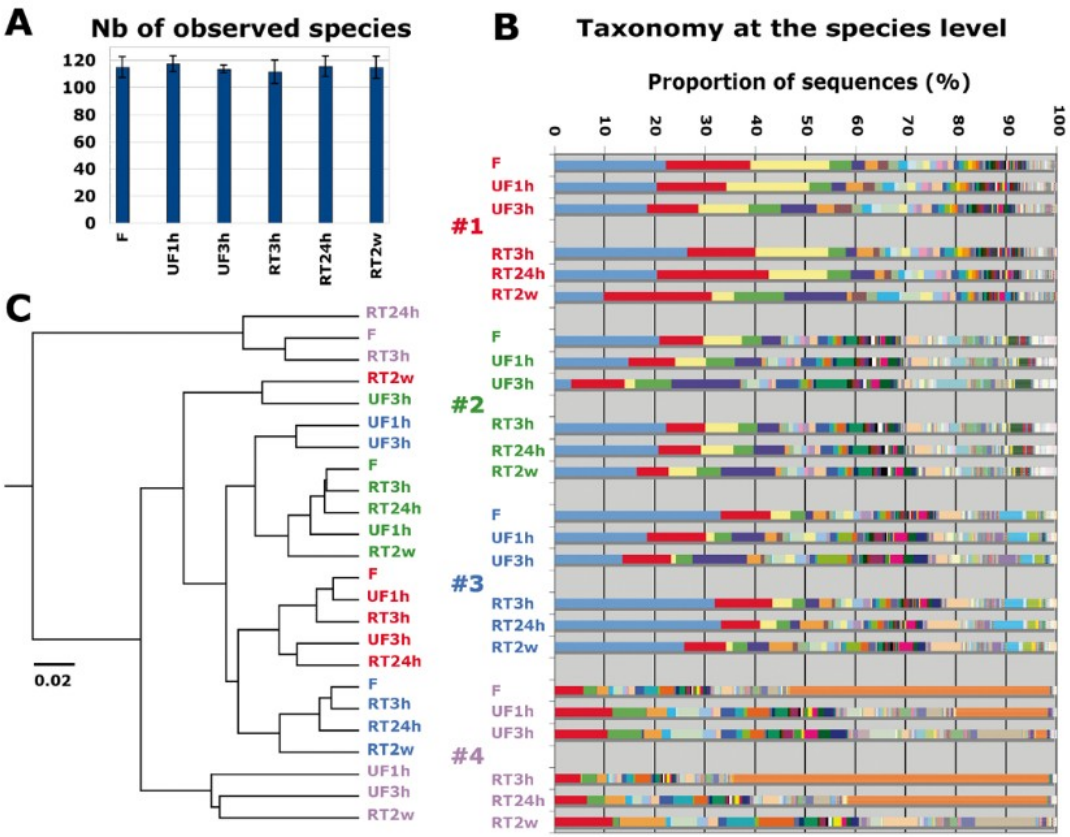


Figure 2 Bacterial community analysis based on 16S rRNA gene survey. A) Alpha-diversity analysis of number of species observed in 6 storage conditions: Immediately frozen (F); unfrozen 1 h and 3 h (UF1h, UF3h); room temperature 3 h, 24 h, and 2 weeks (RT3h, RT24h, RT2w). The plot averages the number of species from the samples provided by 4 individuals in each condition. **B**) Taxonomy analysis at the species level of the 24 samples based on alignment performed using PyNast against Silva 108 release database and OTUs assignment using blast and the Silva 108 release taxa mapping file. Individual #1 (red), #2 (blue), #3 (green), #4 (purple). A more detailed taxonomy assignment is provided in the additional data (See Additional file 3: Table S1). **C**) UPGMA clustering of the 24 samples based on weighted UniFrac method. Samples from the 4 individuals are colored as in B. The scale bar represents 2% sequence divergence.

Vliv laboratorní teploty na degradaci DNA

Table 2 Taxonomic comparison for 3 main bacterial taxa between frozen and unfrozen samples

Taxon	F*	UF1h*	UF3h*	*p* value F vs UF1h	*p* value F vs UF3h
Bacteroides;uncultured bacterium	19	13	9	0.044	9.68e-05
Prevotellaceae;uncultured;human gut metagenome	7	6	3	0.6804	0.0222
Bifidobacterium;uncultured bacterium	2	4	8	0.2257	0.0007

Statistical analysis was performed using Poisson regression model; p value < 0.05 is considered significant; n = 4 subjects; * Values are mean proportion of sequences (%).
F = frozen; UF1h = unfrozen during 1 h; UF3h = unfrozen during 3 h; Taxonomy is indicated at the genus level and if not possible at the family level.

Table 3 Taxonomic comparison for 3 main bacterial taxa between frozen and RT samples

Taxon	F*	RT3h*	RT24h*	RT2w*	*p* value F vs RT3h	*p* value F vs RT24h	*p* value F vs RT2w
Bacteroides;uncultured bacterium	19	20	19	13	0.749	0.749	0.0349
Prevotellaceae;uncultured;human gut metagenome	7	6	5	3	0.6804	0.3189	0.0140
Bifidobacterium;uncultured bacterium	2	2	3	7	1	0.3964	0.0030

Statistical analysis was performed using Poisson regression model. * Values are mean proportion of sequences (%). p-value < 0.05 is considered significant; n = 4 subjects; F = frozen; UF1h = unfrozen during 1 h; UF3h = unfrozen during 3 h; RT = room temperature; 2w = 2 weeks; Taxonomy is indicated at the genus level and if not possible at the family level.

Vliv skladovacích podmínek na bakteriální složení vzorku

SCIENTIFIC REPORTS

OPEN

Sample storage conditions significantly influence faecal microbiome profiles

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Accepted: 13 October 2015
Published: 17 November 2015

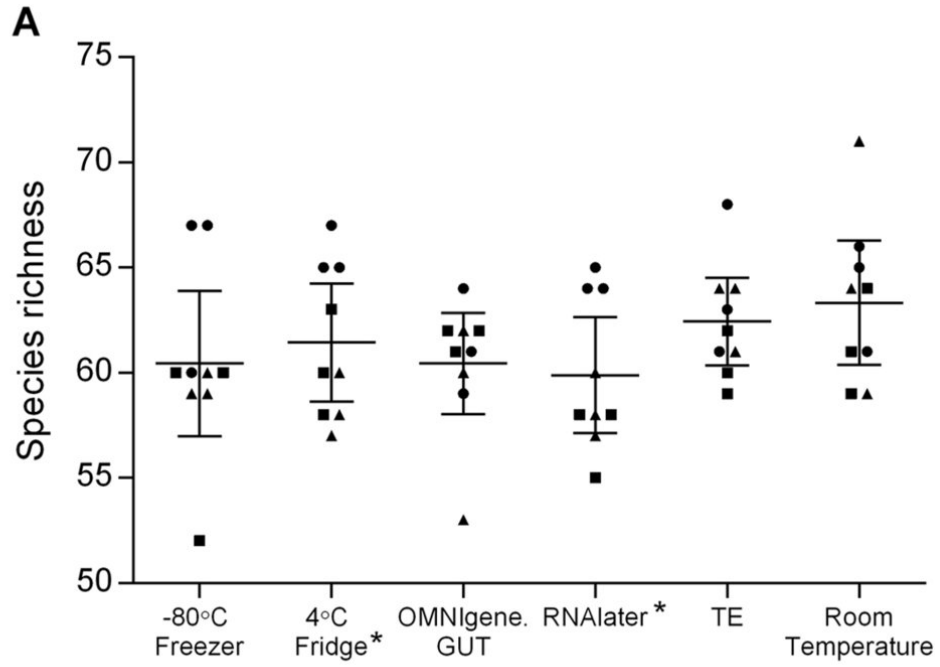
Jocelyn M Choo[1,*], Lex EX Leong[1,*] & Geraint B Rogers[1,2]

Sequencing-based studies of the human faecal microbiota are increasingly common. Appropriate storage of sample material is essential to avoid the introduction of post-collection bias in microbial community composition. Rapid freezing to −80 °C is commonly considered to be best-practice. However, this is not feasible in many studies, particularly those involving sample collection in participants' homes. We determined the extent to which a range of stabilisation and storage strategies maintained the composition of faecal microbial community structure relative to freezing to −80 °C. Refrigeration at 4 °C, storage at ambient temperature, and the use of several common preservative buffers (RNAlater, OMNIgene.GUT, Tris-EDTA) were assessed relative to freezing. Following 72 hours of storage, faecal microbial composition was assessed by 16 S rRNA amplicon sequencing. Refrigeration was associated with no significant alteration in faecal microbiota diversity or composition. However, samples stored using other conditions showed substantial divergence compared to −80 °C control samples. Aside from refrigeration, the use of OMNIgene.GUT resulted in the least alteration, while the greatest change was seen in samples stored in Tris-EDTA buffer. The commercially available OMNIgene.GUT kit may provide an important alternative where refrigeration and cold chain transportation is not available.

Vliv skladovacích podmínek na bakteriální složení vzorku

Figure 1: Species diversity following incubation under six different storage conditions

From: Sample storage conditions significantly influence faecal microbiome profiles

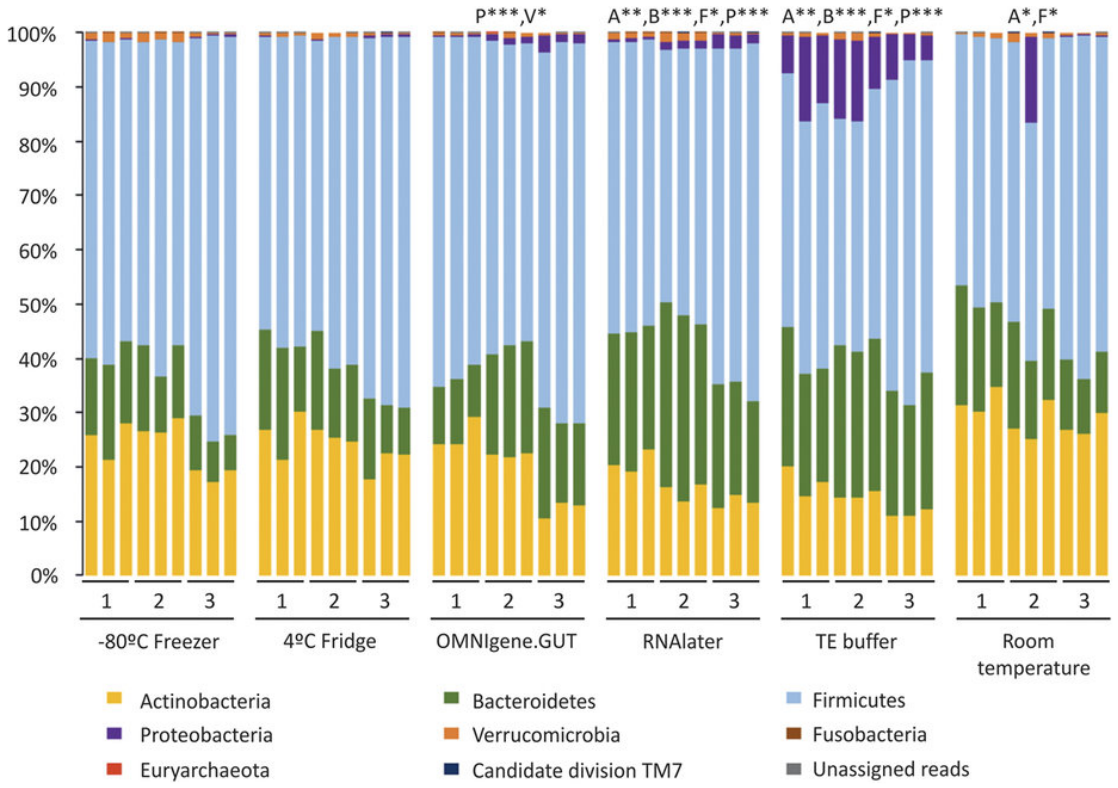


The extent of microbiota structural and composition diversities were measured using (**A**) Taxa S (species richness), (**B**) Shannon-Weiner diversity index, (**C**) Simpson's evenness index. Each point represents the diversity score for a replicate from collection 1 (●), collection 2 (▲) or collection 3 (■). Error bars represent SEM. Within-group and between-group variations were measured using Kruskal-Wallis one-way ANOVA and Mann-Whitney *U*-test, respectively. Significant variance is indicated by asterisks; single asterisk (*) indicates $p \leq 0.05$, double asterisk (**) represents $p \leq 0.01$.

Vliv skladovacích podmínek na bakteriální složení vzorku

Figure 2: Relative abundance at phylum level for each sample incubated under six different storage conditions.

From: Sample storage conditions significantly influence faecal microbiome profiles



Storage conditions that differed significantly from the control (−80 °C) are indicated with respective phylum abbreviation and asterisks. The respective phyla were abbreviated as follow: Actinobacteria (**A**), Bacteroidetes (**B**), Firmicutes (**F**), Proteobacteria (**P**) and Verrucomicrobia (**V**). Statistical significance was assessed by Mann-Whitney U-test and indicated by asterisks; single asterisk (*) represents $p \leq 0.05$, double asterisk (**) represents $p \leq 0.01$, and triple asterisk (***) represents $p \leq 0.001$.

Vliv skladovacích podmínek na bakteriální složení vzorku

Table 1: Mean difference in the relative abundance of the phyla Firmicutes Bacteroidetes, Actinobacteria and Proteobacteria in different storage conditions compared to −80°C.

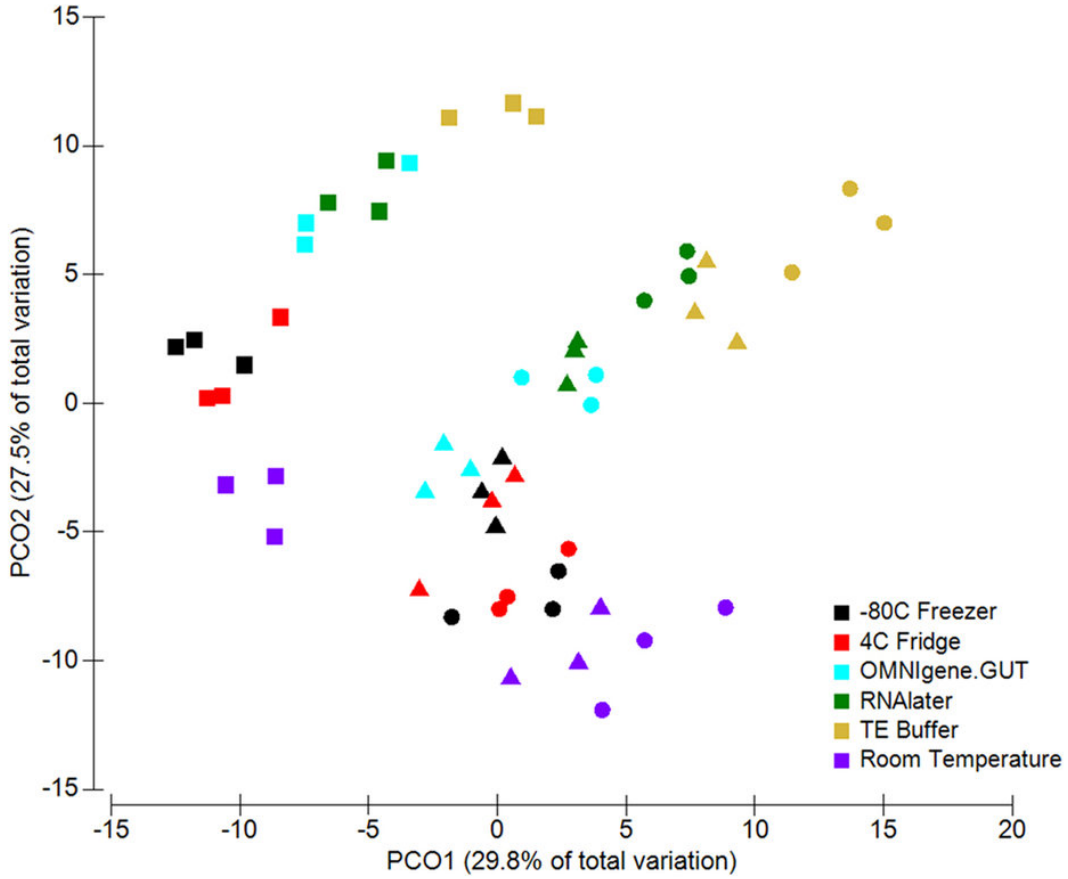
From: Sample storage conditions significantly influence faecal microbiome profiles

	Difference in mean relative abundance ± standard error of mean				
Phylum	**−80 °C vs 4 °C fridge**	**−80 °C vs OMNIgene.GUT**	**−80 °C vs RNA later**	**−80 °C vs TE buffer**	**−80 °C vs RT**
Firmicutes	2.4 ± 1.4	3.6 ± 0.8	7.8 ± 1.6	12.7 ± 1.2	10.5 ± 0.9
Bacteroidetes	2.0 ± 0.5	6.9 ± 1.1	13.6 ± 3.2	12.2 ± 2.4	3.6 ± 0.2
Actinobacteria	1.7 ± 0.3	4.1 ± 1.7	7.0 ± 2.4	9.2 ± 1.7	5.6 ± 2.5
Proteobacteria	0.04 ± 0.02	1.0 ± 0.4	1.3 ± 0.4	10.0 ± 2.3	1.8 ± 1.8

Vliv skladovacích podmínek na bakteriální složení vzorku

Figure 3: Clustering of samples due to storage conditions by PCoA, based on Bray-Curtis similarity distance.

From: Sample storage conditions significantly influence faecal microbiome profiles

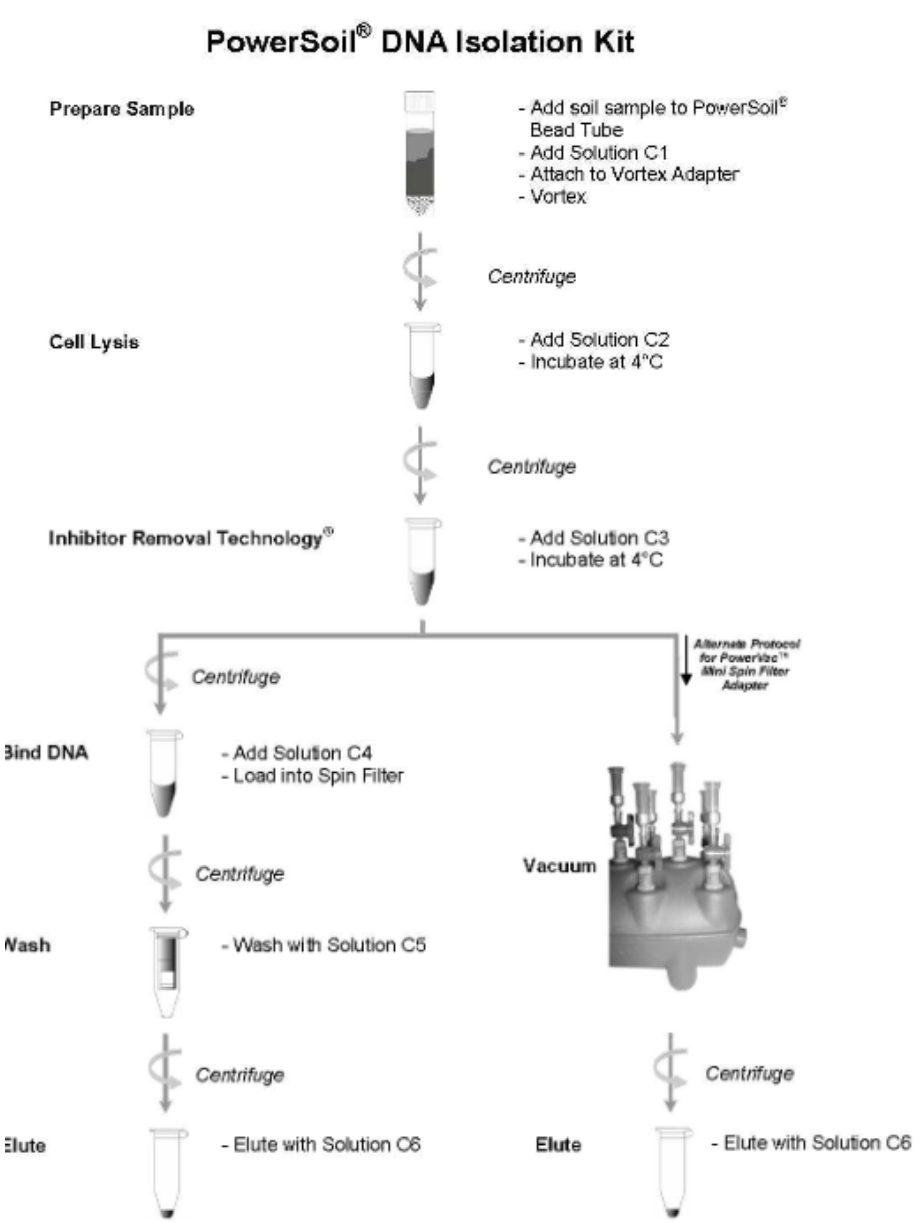


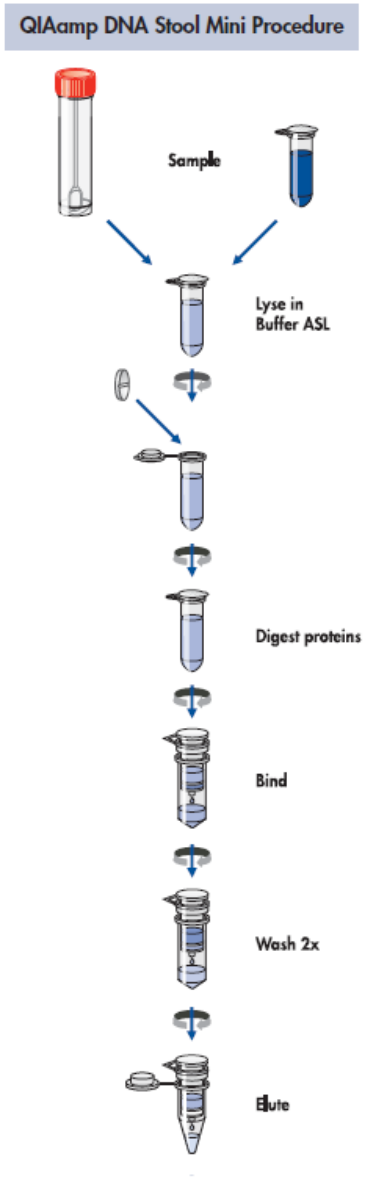
The first two principal coordinates are plotted on the x- and y-axes, respectively (representing 57.3% of the total variation). Faecal collections sampled at three different time points are represented by circle (●) for collection 1, triangle (▲) for collection 2, and square (■) for collection 3. Storage conditions are indicated by colour.

Izolace DNA

- Nejčastěji komerčními kity – přímo dle typu vzorku (MoBio, Qiagen)
- 2 přístupy
  - Lyze buněk enzymaticky
  - Lyze buněk enzymaticky i mechanicky pomocí beat beateru, homogenizátoru →ke vzorku se přidají kuličky s různých materiálů dle výrobce, tento krok lze přidat i u pouze enzymatických kitů
- Doporučuji přidat krok s RNázou A, pokud není součástí postupu

Izolace


PowerSoil® DNA Isolation Kit
Prepare Sample
- Add soil sample to PowerSoil® Bead Tube
- Add Solution C1
- Attach to Vortex Adapter
- Vortex
Centrifuge
Cell Lysis
- Add Solution C2
- Incubate at 4°C
Centrifuge
Inhibitor Removal Technology®
- Add Solution C3
- Incubate at 4°C
Centrifuge
Alternate Protocol for PowerVac™ Mini Spin Filter Adapter
Bind DNA
- Add Solution C4
- Load into Spin Filter
Centrifuge
Vacuum
Wash
- Wash with Solution C5
Centrifuge
Centrifuge
Elute
- Elute with Solution C6
Elute
- Elute with Solution C6


QIAamp DNA Stool Mini Procedure
Sample
Lyse in Buffer ASL
Digest proteins
Bind
Wash 2x
Elute
Fully automatable on the QIAcube

Cíle izolace

- Získat co nejméně degradovanou DNA
  - Degradovaná DNA pro sekvenaci genu 16S rRNA není problém, ale pro scelometagenomové sekvenování už může být – lze řešit vyřezáním z gelu
- Získat co největší množství DNA (opět více důležité pro celometagenomové sekvenování)
  - Pokud není DNA dost – MDA (WGA)