3. Collection and Initial Processing of Faecal Samples

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3.1 Purpose

This protocol discusses the impact that different sample collection methods have on microbiome research and provides examples of methods that have been used at the Quadram Institute for these purposes. It also outlines the initial processing of samples on receipt.

3.2 Introduction

It is integral to microbiome research that the composition of the microbiome in the samples being investigated, is an accurate representation of the composition of the microbiome *in vivo*. It is also essential that sample collection methods are standardised so that results are comparable.

Currently, there is no perfect sample collection method for microbiome studies and each sample collection strategy will have an impact on the viability and diversity of the microbiota subsequently recoverable in the laboratory. For example, faecal samples contain some anaerobic microorganisms that are extremely sensitive to oxygen and may not survive outside the gut in an oxygen-rich environment. This could have huge implications for studies that require the microbiota to remain viable for further applications such as in Faecal Microbiota Transplantation studies. Also, a change in oxygen availability in samples following sample collection, may cause a false positive bias towards aerobic bacteria which could cause misrepresentation of the measured population distribution within the samples.

Therefore, before starting a microbiome research study, it is important to consider which sample collection method is most appropriate for your sample types, the cohort of participants involved in the study (their availability and ability to understand the collection method), your budget, and what outcomes you wish to achieve from your study (DNA/RNA studies only, or phenotypic investigations such as culturing).

In a perfect world, once a sample has been taken, all downstream analysis would be done immediately without any need for storage or transportation. Unfortunately, this is not feasible for most microbiome studies and participants will usually collect their samples at home. Where immediate processing of samples is not possible, freezing at -80°C is widely considered to be best practice when attempting to maintain microbial composition in the samples for sequencing and culturing applications [1, 2, 3]. However, storage at -80°C is not always practical if participants are collecting samples away from the research facility. Where storage at -80°C is not possible, storage of samples at 4 °C (preferably lower) can minimise any change in microbial composition [3, 4, 5].

In order to ensure participant compliance in sample collection methods, the sample collection method must be straightforward and convenient so that the participant can easily send off their sample to the research facility.

In this document, we will describe some options for sample collection methods, several of which have been used by the Quadram Institute during their human studies where microbiome samples were required from participants.

3.3 Materials

3.3.1 Faecal sample collection kit

There is no perfect sample collection kit for microbiome research. Each has its advantages and disadvantages and a decision on what would be most suitable should be based on each individual project. However, the basic attributes of a faecal sample collection kit are shown in Table 1.

Table 1. Materials required for faecal sample collection kits

Sample collection kits				
	Item	Requirements	Example	
			Product	Supplier
Basic items to include	Participant sample collection instructions	- Easy to understand - Include pictures and diagrams	Developed in house (Annex 1 and 4)	Developed in house
	Sample collection log	- for participants to record time and date of sample collection	Developed in house (Annex 2 and 6)	Developed in house
	Sample collection device	- Able to catch stool sample without contaminating the sample - Must be easy and hygenic to use - Must be easy to dispose of	FecesCatcher	Tag Hemi VOF
	Sample container	- Must be able to contain samples of all consistencies - If trying to create anaerobic conditions, the sample container must be able to maintain these conditions	Sterilin™ Polystyrene 30mL Universal Containers	Many
Additional items to consider	Items to maintain an anaerobic environment	- Must be compatible with the sample container - the outer layer must be airtight to produce the anaerobic environment, but the sample must be exposed to the atmosphic environment of the anaerobic gas generating sachet - Must check the capacity of the sachet will work for the size of your	Thermo Scientific™ Oxoid™ AnaeroGen™ 2.5L Sachet	Fisher Scientific
	Items to maintain cold chain transfer to the laboratory	- To maintain samples at a cool temperature until processed the item required will depend on the method of sample collection being utilised, and how long it will be until the sample is transported to the laboratory after collection.	Diagnosach	Tempack
	Items to deliver sample to the laboratory	- This will depend on the sample collection method being used, if the participant is bringing the sample to the laboratory they may need a small cool box, whereas if they are posting it or using a courier, this must meet local regulations on transporting biological samples.	Royal Mail Safebox®	Royal mail

For full procedures used at the Quadram Institute in the PEARL and MOTION studies, please refer to Annex 3 (MOTION), 5 and 7 (PEARL)

3.3.2 Initial processing of faecal samples

Table 2 describes the items required specifically for the initial processing of faecal samples collected for the PEARL and MOTION studies at the Quadram Institute. For full details on initial processing please refer to Annex 8 (MOTION) and 9 (PEARL).

Table 2. Materials required for initial processing of faecal samples

Initial sample processing		
Item	**Requirements**	**Product**
Sample receipt log	- Include participant ID, the timepoint of the sample (if multiple for the study), the sample type (if multiple for the study), the time and date that the sample was collected, the time and date that it was received and processed, the number of aliquots made, the weight of the aliquots made, a space for notes to comment on sample quality etc.	Developed in house
Cryovials	- Must be suitable for cold storage and be compatible with freezer storage systems (boxes and shelving) - Externally threaded is preferable - Must be sterile	1.8 ml Cryovial with External Thread, Self-Sealing Cap, Skirted (Sterile)
Tubes with glass/ ceramic beads and spheres (to be later used in DNA extraction processes)	- Must be sterile - Must be compatible with equipment that will be used to bead beat the sample prior to DNA extraction	Lysing Matrix E, 2mL tubes
Freezer boxes	- Must be suitable for freezers and compatible with the cryovials being used	Polypropylene Microtube Storage Box
Racks to hold samples while processing	- Must be compatible with the cryovials/ tubes with beads - Can be cleaned easily	Polypropylene Micro Tube Rack
Sterile inoculating loops (to transfer stool into crovial)	- Not too flexible or it can flick sample - Disposable	10ul Inoculating Loops (50 per sleeve)
Precision Balance	- Must be capable of measuring down to 1mg	Precision Balance MS1003TS/00
Suitable Personal Protective Equipment (PPE)	- A local risk assessment should identify the protective equipment necessary for the procedures being undertaken. - Example items include lab coat, protective gloves, protective eyewear etc.	Many
Biological Safety Cabinet	- Must offer at least class 2 level protection	Any Class 2 Biological Safety Cabinet
-80°C freezer	- Must have capacity to store all samples for your study - Monitoring system for freezer temperature is an advantage here	Any -80°C freezer

3.3.3 Further processing

- **Water content of faecal samples**

Below, in Table 3 are details of the items required to conduct a basic analysis of water content of faecal samples using a freeze drying method. For exact information of items used for the MOTION and PEARL studies, please refer to Annex 11.

Table 3. Materials required for determining water content of faecal samples

Water content of faecal samples		
Item	**Requirements**	**Product**
Precision Balance	- Must be capable of measuring down to 1mg	Precision Balance MS1003TS/00
Freeze drier	- Must be specific to your volume requirements	ModulyoD Freeze Dryer – 5 Liter
Parafilm	n/a	Many
Pre-weighed faecal sample	n/a	n/a
Suitable Personal Protective Equipment (PPE)	- A local risk assessment should identify the protective equipment necessary for the procedures being undertaken. - Example items include lab coat, protective gloves, protective eyewear etc.	Many

- **Absolute quantification of microbes in human faecal samples**

Table 4 describes the basic materials needed to quantify microbes in faecal samples via flow cytometry. For specific information about what is used in the PEARL/MOTION studies, please refer to Annex 12

Table 4. Materials required for absolute quantification of microbes in human faecal samples

Absolute quantification of microbes in human faecal samples		
Item	**Requirements**	**Product**
SYBR green stain	n/a	n/a
PBS	n/a	n/a
Flow cytometer	n/a	n/a
Computer	- Must have compatible software with the flow cytometer to analyse data	n/a
Consumables	- Must be specific to the flow cytoometer being used, refer to user manual	n/a
Bleach	- Concentration must be specific to flow cytometer and advice provided in user manual	n/a
Pre-weighed faecal sample	n/a	n/a
Suitable Personal Protective Equipment (PPE)	- A local risk assessment should identify the protective equipment necessary for the procedures being undertaken. - Example items include lab coat, protective gloves, protective eyewear etc.	Many

- **DNA extraction**

This is mentioned in initial processing methods and covered in detail in Section 4: DNA Extraction and Quality Control. For a short introduction, please refer to Annex 10.

3.4 Methods

3.4.1 Sample collection procedures

Table 5 shows examples of previous sample collection kits and procedures used at the Quadram Institute for human microbiome studies and explains the key considerations for each. For full details of the sample collection methods used in the PEARL and MOTION studies, please refer to Annex 3 (MOTION), 5 and 7 (PEARL).

Table 5. Examples of faecal sample collection kits used in human microbiome studies at the Quadram Institute

Example faecal sample kits used in human microbiome studies at the Quadram Institute					
Kit	**Overview**	**Contents**	**Why necessary**	**Considerations for which type to purchase**	**Key considerations**
Faecal sample collection kit 1 (MOTION study)	This sample collection kit was designed to maintain the sample at 0°C or lower for 24 hours with an atmosphere with reduced oxygen content. This kit was used in a study where participants were asked to produce a stool sample at home within 24 hours of a pre-booked appointment at the research facility. The participant would bring their sample with them and hand it to the research team on arrival where it would be pre-processed and stored at -80°C.	Gloves	To ease participants' minds for sample collection	Must check gloves are suitable for allergies for participants. Latex gloves should not be used for this reason.	**Ease:** Although this procedure is not very technical, there are lots of aspects to the sample collection and a very detailed, step by step guide for participants is essential to ensure compliance and consistency of sample collection. **Cost:** This is a medium to high cost sample collection method depending on the options of cool box and stool collection device selected. There are, however, no delivery costs as the participant brings the sample with them to their appointment. **Sample integrity:** Samples are delivered to the research facility within 24 hours of being produced while being stored at 0°C or lower for 24 hours in an atmosphere with reduced oxygen content, therefore, sample integrity is reasonably high in comparison to many stool sample collection techniques.
		Stool collecting device	For ease of sample collection for participants, and to encourage the collection of quality samples	Costs can vary massively in different variations of collection devices. Some are much larger than others so could have problems in terms of storage and posting to participants. Some models are biodegradable and can be flushed down the toilet after sample collection making it easier and more hygienic for participants to dispose of.	
		Sample bag	There is a need for something to contain the sample.	For this kit, the bag must remain open so that the anaerobic gas generating sachet can work	
		Anaerobic gas generating sachets	To reduce oxygen in the immediate surrounding of the sample	Must check volume to ensure it provides an anaerobic environment in	
		Air tight outer packaging	To produce an anaerobic environment	This must be air tight to ensure an anaerobic environment is maintained	
		Sample collection log	To accurately record time and date of sample collected	n/a	
		Cool box	To maintain temperature of samples at 0°C or lower for 24 hours	Must be convenient and light enough for sample transport to the laboratory. Must be able to maintain the samples at 0°C or lower for 24 hours	
		Cool packs	To maintain temperature of samples at 0°C or lower for 24 hours	Must be convenient and light enough for sample transport to the laboratory. Must be able to maintain the samples at 0°C or lower for 24 hours	

Faecal sample collection kit 2 (PEARL study)	This sample collection kit was used in a study where we collected a number of sample types so the process needed to be very straight forward. The cohort were pregnant women from early in their pregnancy and following them and their infant until the infant is 2 years old. The study is completely remote with the participants not needing to attend the research facility. The cohort are going through a notoriously busy phase in their lives so we had to ensure that the delivery method to the research facility was as easy for the participants as possible. Therefore, we have provided participants with a small benchtop freezer where they can store their samples at home immediately after they collect their sample using the collecting device and scooping it into the sample collection pot using the inbuilt spoon. The research team then collect samples from the research participants and transport samples back to the research laboratory using cold chain methods.	Stool collecting device	For ease of sample collection for participants, and to encourage the collection of quality samples	See above	**Ease:** This is a very straight forward sample collection method and is incredibly convenient for participants as they can store their samples at home until a convenient time for them when the research team picks up the samples from them. **Cost:** There is an initial investment required in the benchtop freezers for participants. **Sample integrity:** As the participant has a freezer where they can store their samples immediately after they have been produced, sample integrity is very high. Cold chain can be maintained when the research team collect the samples.
		20mL universal container with spoon.	To contain the sample until back at the research laboratory	n/a	
		Biohazard bag	To provide another level of containment for samples and to hold multiple samples from the same timepoint together in the sample freezer provided to the participant	n/a	
		Benchtop freezer	To put samples in immediately after collecting until the research team can collect the samples from the participants' house	The freezer must be energy efficient and small enough to be accommodated by the participants.	
		Sample collection log	To accurately record time and date of sample collected	n/a	
Faecal sample collection kit 3 (BAMBI study)	This sample collection kit was designed with the main priority being ease of participation in the study. The study involved collecting stool samples from premature infants while they were in the hospital, and then when they were at home. This kit was used specifically for when the participants were at home.	20mL universal container with spoon.	To contain the sample until back at the research laboratory	n/a	**Ease:** This is a very simple procedure for participants. There are no complex procedures to follow and the samples can be put in their local postbox at their convenience. **Cost:** This is a very low cost sample collection method due to the minimalistic procedures. **Sample integrity:** It is difficult to provide an exact timeframe of delivery of samples. There are also limitations as the service is only on working days which could lead to delays in delivery; there is no cold chain meaning samples could be in very warm conditions during the summer months. However, if participants are fully briefed to not take samples on/near the weekend, then samples should be delievered within 24 hours, which should have only a small impact on sample integrity.
		Royal Mail Safebox®	To provide an all included packaging system for up to 50mL biological sample to UN3373 standards using the normal postal service.	There are two options available: First Class Safebox™ - with a delivery target of next day, which can be put in any post-box. Special Delivery™ - Guaranteed next working day delivery, but needs to be taken to a Post Office.	

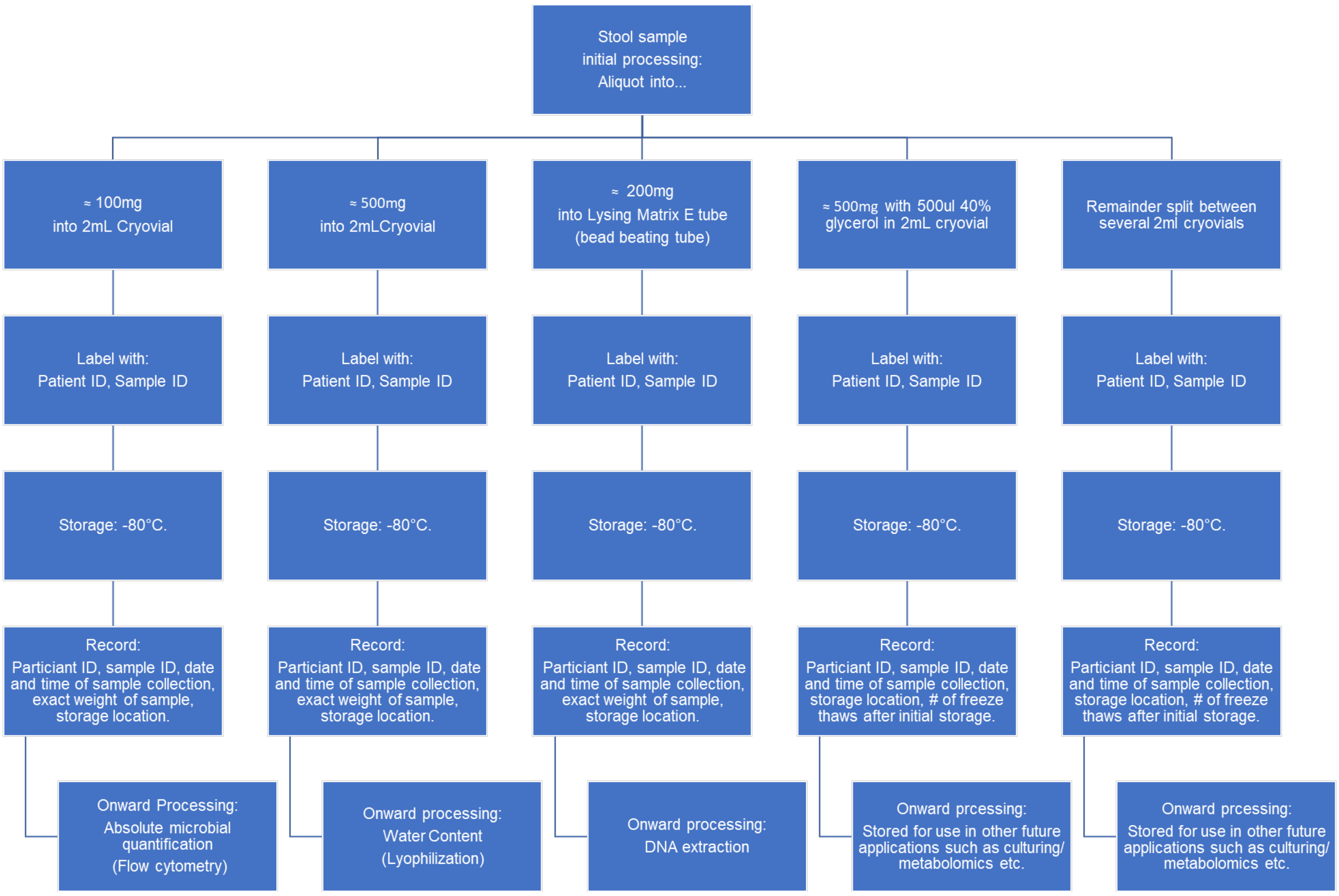
There are several other options that may be of interest for microbiome research.

If you are only considering DNA/RNA applications from your samples there are products that allow you to immediately preserve the nucleic acids within samples at the time of collection for subsequent microbiome profiling. They usually involve a small tube (usually about 5 mL) with a spatula or spoon filled with stabilisation buffer. Once samples are in the buffer, they remain stable for long periods of time at ambient temperatures (some report > 2 years), which reduces the requirement for low temperature storage. Kits vary, but some are provided with a faecal collection device and packaging materials for postage. These kits do have limitations as you will receive a much smaller volume of sample (one of these devices reports 520 +/- 101mg of faeces per collection kit). The microorganisms in these samples are inactivated and so would no longer be viable for use in applications such as culturing. Two examples of these devices are Zymo Research DNA/RNA Shield and OMNIgene®•GUT.

Another option involves faecal collection pots with an in-built chamber containing a reagent that produces anaerobic conditions. This maintains the anaerobic conditions and increases the survival of extremely oxygen-sensitive microorganisms within the sample. Products claim that a period of up to 72 hours at room temperature between sample collection and delivery to the laboratory is possible without any significant reduction in bacterial viability; it is recommended that samples are frozen to preserve high abundances of the microbial populations within the sample. Currently, these devices are quite expensive compared with other types of sample pots (one company has quoted 50€ per kit). However, by reducing the requirement (and expense) of maintaining a cold chain for samples, they are also quite unique in what they currently offer. If it is important to maintain viability of samples for further processing, such as for FMT or culturing work, it may be worth considering working with samples in an anaerobic cabinet. An example of this device is the GutAlive®.

3.4.2 Initial sample processing

Samples received at the Quadram Institute from the PEARL and MOTION studies undergo immediate sample processing. This initial processing step divides the sample into ready-to-use aliquots for our standard processing (water content, DNA extraction, absolute quantification) and provides us with several smaller aliquots of the sample for other future applications such as culturing. This is very useful as once the sample is frozen at -80°C, it is very difficult to manipulate. It is also best practise to reduce how many times a sample is frozen and then thawed to prevent any degradation of sample integrity. The following flow chart explains how we initially process our faecal samples:

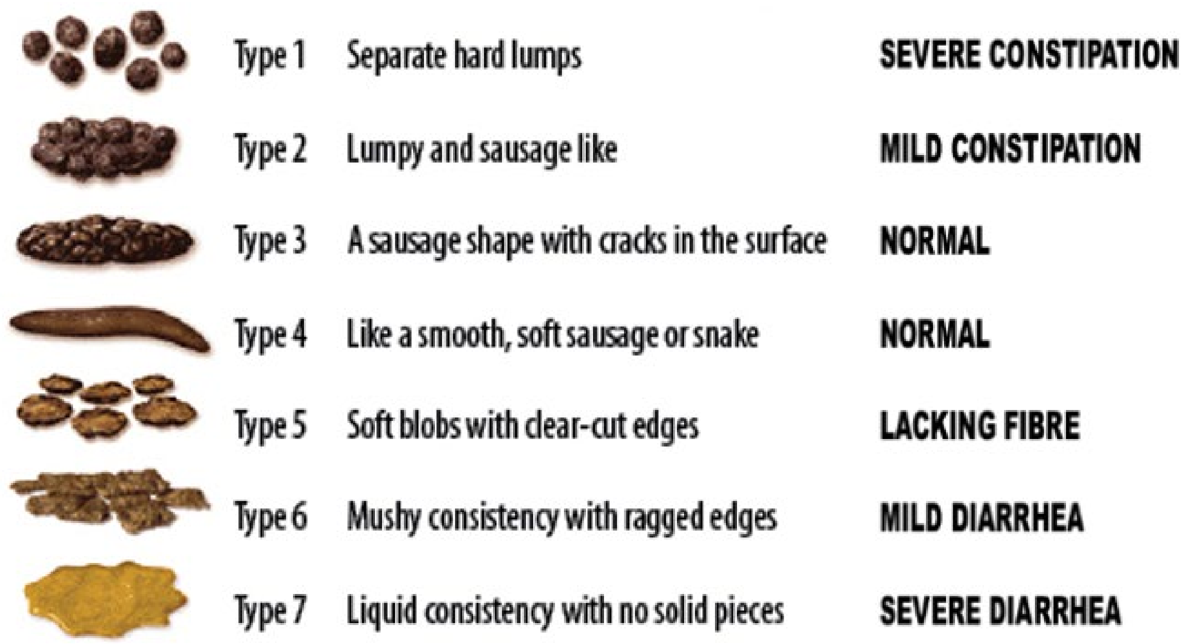


For full details of initial processing please refer to Annex 8 (MOTION) and 9 (PEARL).

3.4.3 Further processing steps

- **Bristol Stool Chart Classification**

For each sample collection, participants are shown a copy of the Bristol Stool Chart, a clinical assessment tool designed to classify faeces (stool) into seven groups. This scale is a useful tool to determine whether the sample is from a normal or an abnormal bowel movement, according to the participants' assessment.



Cabot Health, Bristol Faecal Chart - http://cdn.intechopen.com/pdfs-wm/46082.pdf

- **Determining water content of faecal samples by lyophilization**

A healthy gut microbiome characterised by high bacterial species richness and diversity, reflecting stability and resilience of the microbial ecosystems of which it is comprised [6, 7].

The water content of faeces can tell you a lot about a sample, for instance, its transit time through the intestinal colon. The longer the faeces takes to pass through the intestinal colon, the more water is reclaimed back into the body and the dryer the faeces becomes. It can be affected by diet (particularly fibre intake), medications and physical inactivity.

Transit time acts as a selective force on gut bacterial growth rates. Faecal consistency strongly correlates with all known major microbiome markers and is negatively correlated with species richness [8].

Lyophilisation (freeze drying) removes water from a sample by first freezing the sample, then reducing the pressure and warming to allow the frozen water in the material to sublimate (convert straight from a solid form to a vapour on heating). This method can be used to determine the original water content of a sample by comparing the weight of the sample before and after freeze drying to determine the percentage of the sample that was originally water. All our samples are analysed for their water content by lyophilization as follows:

It is important to record all weights, i.e. the weight of the cryovial, the exact weight of the faecal sample after it has been aliquoted into the cryovial, and finally the weight after lyophilisation.

Lyophilization protocols will differ depending on which model of freeze dryer is used. Our freeze dryer requires us to process our samples for 12 hours.

The water content of faeces should be calculated by subtracting the freeze-dried faecal weight from the weight of the faeces before freeze drying and converting this into a percentage of the original sample weight.

For exact information about the materials used for this in the MOTION and PEARL studies, please refer to Annex 11.

- **Absolute quantification of microbes in human faecal samples**

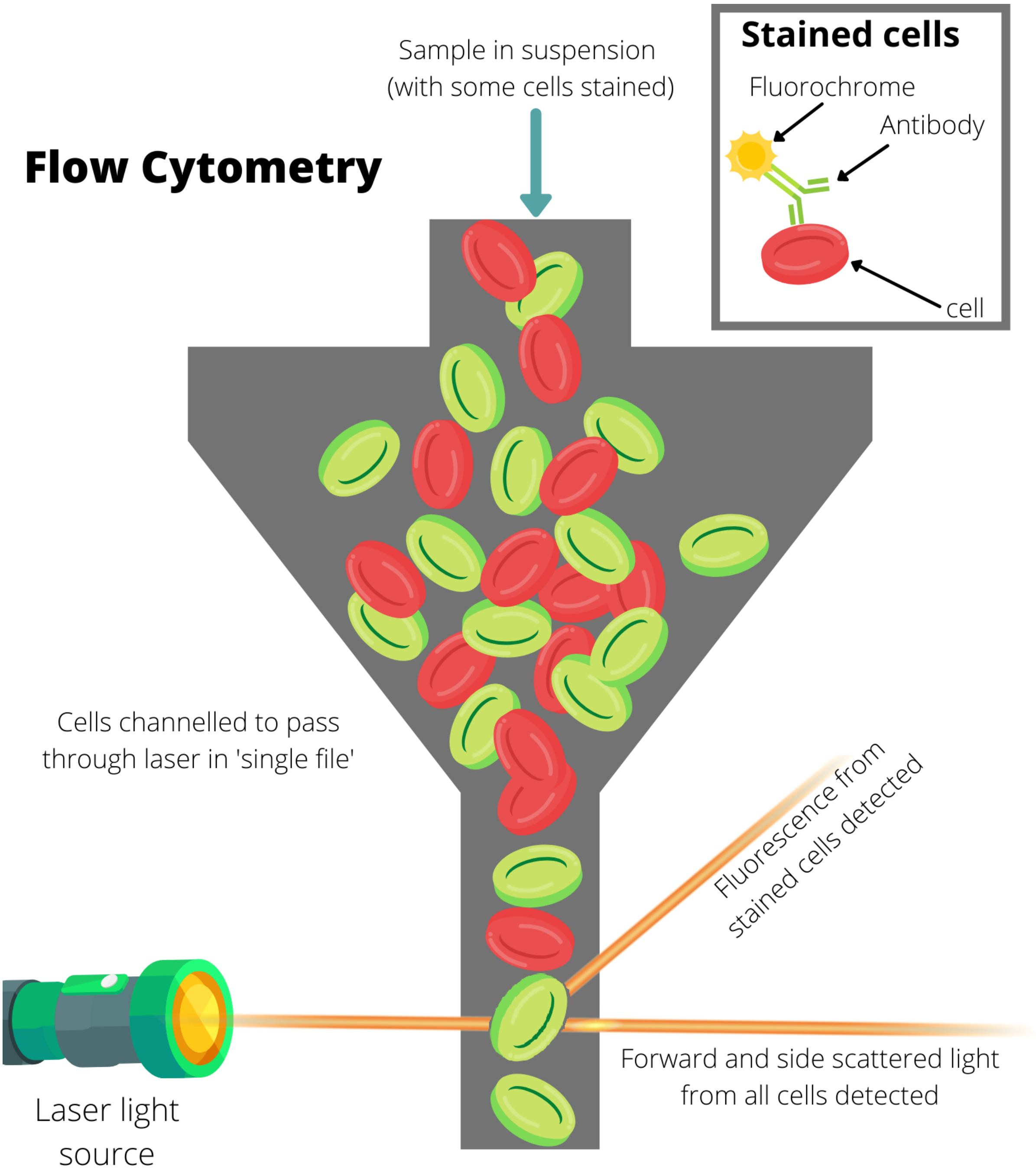
Current comparative sequence-based analyses of faecal microbiota are limited in their ability to provide information about the extent or directionality of changes in the abundance of taxa or their metabolic potential and, therefore, to reveal the interplay between microbiota and host health.

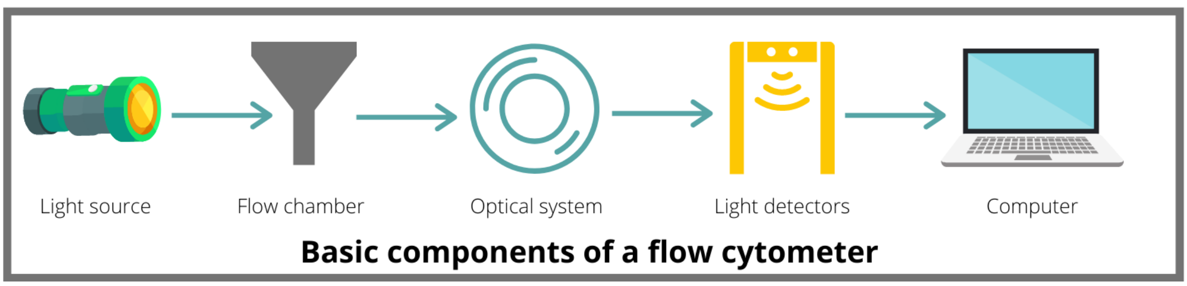
Microbial load has been identified as a key driver of observed differences in the microbiota present in certain cohorts of patients, such as those with Crohn's disease, compared with healthy individuals.

Therefore, the samples from our two longitudinal studies, PEARL and MOTION, are processed by flow cytometry to enable quantitative microbiome profiling of faecal samples by parallelisation of amplicon sequencing and flow cytometric enumeration of microbial cells.

Flow cytometry is an analytical tool that rapidly detects and characterises cells in a liquid suspension of a sample. The instrument organises the sample into a single-file stream of cells that pass a beam of light (usually a laser). As the light hits each cell, light is scattered in

a way that is characteristic of the physical structure of the cell. Depending on where the scattered light is detected by the flow cytometer, these measurements are termed ‘forward’ and ‘side’ scatter. If a sample has been stained with fluorophores, the laser will excite these and a fluorescent emission will be produced and detected. This information is sent to a computer where the data can be analysed by the user (see overview below).
Microbes can be detected by staining them with a fluorochrome (such as SYBR green).





In the PEARL and MOTION studies, we use the Luminex Guava Easycyte instrument which is a high throughput sample processor that can detect particles as small as 0.2 um, enabling us to investigate the bacterial populations within clinical samples collected from both studies. However, other platforms could also MAKE these analyses.

We stain faecal samples with SYBR green, to identify the microbes in the sample, and measure the number of microbes per gram of faeces.

The general process for absolute quantification of microbes in human faecal samples involves:

This protocol varies depending on the instrument being used. For detailed information of processing samples on the Guava EasyCyte using the PEARL/MOTION study protocols in Annex 12, developed by K. Seton.

3.5 Tips and Troubleshooting

The sample collection method chosen will depend on a variety of factors including; the cohort of participants involved in the study (their availability and ability to understand the collection method); what outcomes you wish to achieve from the study (DNA/RNA studies only, or phenotypic investigations requiring culturing). See discussion in introduction and materials and method section for more details.

References

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8. Vandeputte D, Falony G, Vieira-Silva S et al. (2016) Stool consistency is strongly associated with gut microbiota richness and composition, enterotypes and bacterial growth rates. Gut 65:57-62.

Annex List

Sample collection kit and sample transport to the laboratory	Annex 1	MOTION	Participant instructions for faecal sample collection	Annex 13 Version 3 13.12.2018 Participa
	Annex 2	MOTION	Faecal sample collection log	Annex 14 Version 2 13.12.2018 Participa
	Annex 3	MOTION	Items for faecal sample collection during study visits	241617-SC-007 MOTION study_Item
	Annex 4	PEARL	Participant instructions for faecal sample collection	Annex 8 V4 07.12.2018 Participa
	Annex 5	PEARL	Supply of collection packs for PEARL study participants	241880-LH-004 PEARL study_Supply
	Annex 6	PEARL	Faecal sample collection log	Annex 9.4 V3 10.06.2020 Participa
	Annex 7	PEARL	Collection of faecal samples from participants and supplying collection packs	241880-LH-005 PEARL study_Collect
Initial processing	Annex 8	MOTION	Faecal sample receipt, handling, storage and disposal	241617-SC-006 MOTION STUDY SAN
	Annex 9	PEARL	Faecal sample receipt, handling storage and disposal	241880-LH-007 PEARL study_Sample
Further processing	Annex 10	PEARL and MOTION	Faecal sample DNA extraction using the Promega Maxwell	241617-SC-024 MOTION study_ Sto
	Annex 11	PEARL and MOTION	Determining water content of faecal samples by freeze drying (wet weight)	241617-SC-027 MOTION study_dete
	Annex 12	PEARL and MOTION	Absolute quantification of microbes in human faecal samples	Absolute quantification of mi