Review

Preanalytical Processing and Biobanking Procedures of Biological Samples for Metabolomics Research: A White Paper, Community Perspective (for "Precision Medicine and Pharmacometabolomics Task Group"—The Metabolomics Society Initiative)

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BACKGROUND: The metabolome of any given biological system contains a diverse range of low molecular weight molecules (metabolites), whose abundances can be affected by the timing and method of sample collection, storage, and handling. Thus, it is necessary to consider the requirements for preanalytical processes and biobanking in metabolomics research. Poor practice can create bias and have deleterious effects on the robustness and reproducibility of acquired data.

CONTENT: This review presents both current practice and latest evidence on preanalytical processes and biobanking of samples intended for metabolomics measurement of common biofluids and tissues. It highlights areas requiring more validation and research and provides some evidence-based guidelines on best practices.

SUMMARY: Although many researchers and biobanking personnel are familiar with the necessity of standardizing sample collection procedures at the axiomatic level (e.g., fasting status, time of day, "time to freezer," sample volume), other less obvious factors can also negatively affect the validity of a study, such as vial size, material and batch, centrifuge speeds, storage temperature, time and conditions, and even environmental changes in the collection room. Any biobank or research study should establish and follow a well-defined and validated protocol for the collection of samples for metabolomics research. This protocol should be fully documented in any resulting study and should involve all stakeholders in its design. The use of samples that have been collected using standardized and validated protocols is a prerequisite to enable robust biological interpretation unhindered by unnecessary preanalytical factors that may complicate data analysis and interpretation.

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The preanalytical steps in specimen acquisition and processing are among the most crucial for producing reliable analytical results and are highly vulnerable to the introduction of experimental bias and variance. Such steps must be conducted rigorously to preserve the intrinsic value of the resulting specimen repository or biobank. Biobanks are facilities where carefully collected and curated biological samples are stored and distributed for research purposes according to approved protocols. The samples themselves are linked to metadata, such as age, sex, ethnicity, health status, and potentially medical records of the sample donor.

The number of biobanks around the world has increased *(1)*, reflecting the growing awareness of the need for epidemiological scaled "omics" analyses. This growth has been organic rather than structured, leading to several differing sample collection and processing protocols. Increasingly, there have been attempts (e.g., International Agency for Research on Cancer biobank, Promoting Harmonization of Epidemiological Biobanks in Europe, Biobanking and Biomolecular Resources Research Infrastructure) *(2–4)* to harmonize and rationalize multicentric sample collection and storage protocols. This reduces interbiobank variability, thus improving comparability of observations seen between multicenter projects.

Most existing biobanks were established for epidemiological purposes with either genomics or more traditional scientific analyses in mind; however, the collection requirements for DNA extraction and analyses are fundamentally different than those of metabolomics because DNA extraction is comparatively more robust (e.g., it is still possible to achieve high yields of representative good-quality DNA from blood even after 24-h storage at room temperature) (5). By comparison, metabolism can continue in tissues or biofluids even after collection. For subsequent measurements to be meaningful, the metabolome at the time of extraction and analysis should reflect as closely as possible the original in vivo metabolome. During the sample collection, the concentrations of metabolites may change because of ongoing enzymatic activities, exposure to oxygen, UV light, or temperature (6–8). Protocols must reduce such effects to a minimum and, when unavoidable, standardize exposure of samples to such factors. Importantly, protocols should provide evidence of experimental validation, especially robustness and reproducibility of data for all protocols designed to collect and store metabolomics samples. Fully validated protocols (8, 9) enhance confidence among researchers and promote a gradual rationalization of protocols toward "best practice." We define a validated collection protocol as one where all steps have been tested for reproducibility and robustness specifically on the sample types being collected, with an effort to optimize these steps, including, wherever possible, well-planned comparisons with alternative methods. There should also be a documented awareness of the limitations of the protocol in place, especially when compromises have been made because of practical considerations.

There are 3 key considerations for any sample collection used in metabolomics research:

- Samples should be collected in a reproducible manner within and between different collection sites using the same detailed protocols and the same consumables (including brand and preferably batch number).
- Collected and stored samples should represent the in vivo situation as closely as possible.
- The practicalities of sample collection should be considered in protocol design so that sufficient numbers of samples can be collected with ease.

A schematic of the various stages of the biobanking process is shown in Fig. 1.

Variables that have been shown to affect the measured metabolome in a variety of tissue and biofluid types include age, sex, weight, whether breast-fed (infants), diet, ethnicity, smoking history, environment, time of day, time of year, fasting state, current medications (including nonprescription medications and dietary supplements), physical activity, occupation, and geographic location (10–20). These details, along with a sample of

stool or information on the gut microbiome, should always be recorded when biobanking samples and, when possible, standardized within any individual study. For women, recording the day of her menstrual cycle (and any chemical contraception being used) should be routine because it may affect the metabolome of some biofluids (e.g., as observed in plasma) (10).

Samples should be stored as multiple representative aliquots, when possible, because multiple freeze–thaw cycles may be detrimental to individual metabolites (21). To be considered representative, an aliquot must be taken from a freshly mixed sample because of the tendency of biofluids to settle quickly. Consideration should be given to the size of the aliquot and the size and type of vial it will be stored in, as a large dead space volume can affect sublimation of CO_2 in long-term storage (9) and certain vial materials are known to contaminate samples (22).

A design consideration for sample collection that is often overlooked but is of vital importance is the decision as to whether targeted or untargeted analysis is to be performed. Untargeted analysis would generally seek the greatest molecular feature diversity and the features most reflective of the specimen. Sample collection and processing would be devised accordingly. Targeted analyses may be willing to sacrifice breadth and diversity of molecular features in favor of preselected analytes of interest and optimize the processing and analytical methods best suited for their measurement. A variation on this would be the use of targeted analysis to capture analytes of interest, while simultaneously examining the untargeted metabolome to provide context (23). In such cases, sample acquisition and processing methods that optimize for both needs may be warranted.

In this review, we examine current practice in biobanking samples for metabolomics and the evidence base behind the choices that have been made in defining the protocols, and make recommendations as to the need for further research. A collection of protocols in use for different tissue and biofluid types is summarized in Tables 1, 2, and 3.

Statistical Considerations

It is important to emphasize the importance of validated biobanking protocols with respect to the concept of robust and reproducible scientific discovery, and the statistical inference on whether an observed effect is real, a result of random chance, or a statistical error. Discovery through random chance has been discussed previously and, therefore, is not included (24) in this review; however, false discovery owing to statistical error is often the result of inconsistent or biased sample collection. Inconsistent (imprecise) sample collection can increase biological variance to the point that a given study is underpow-
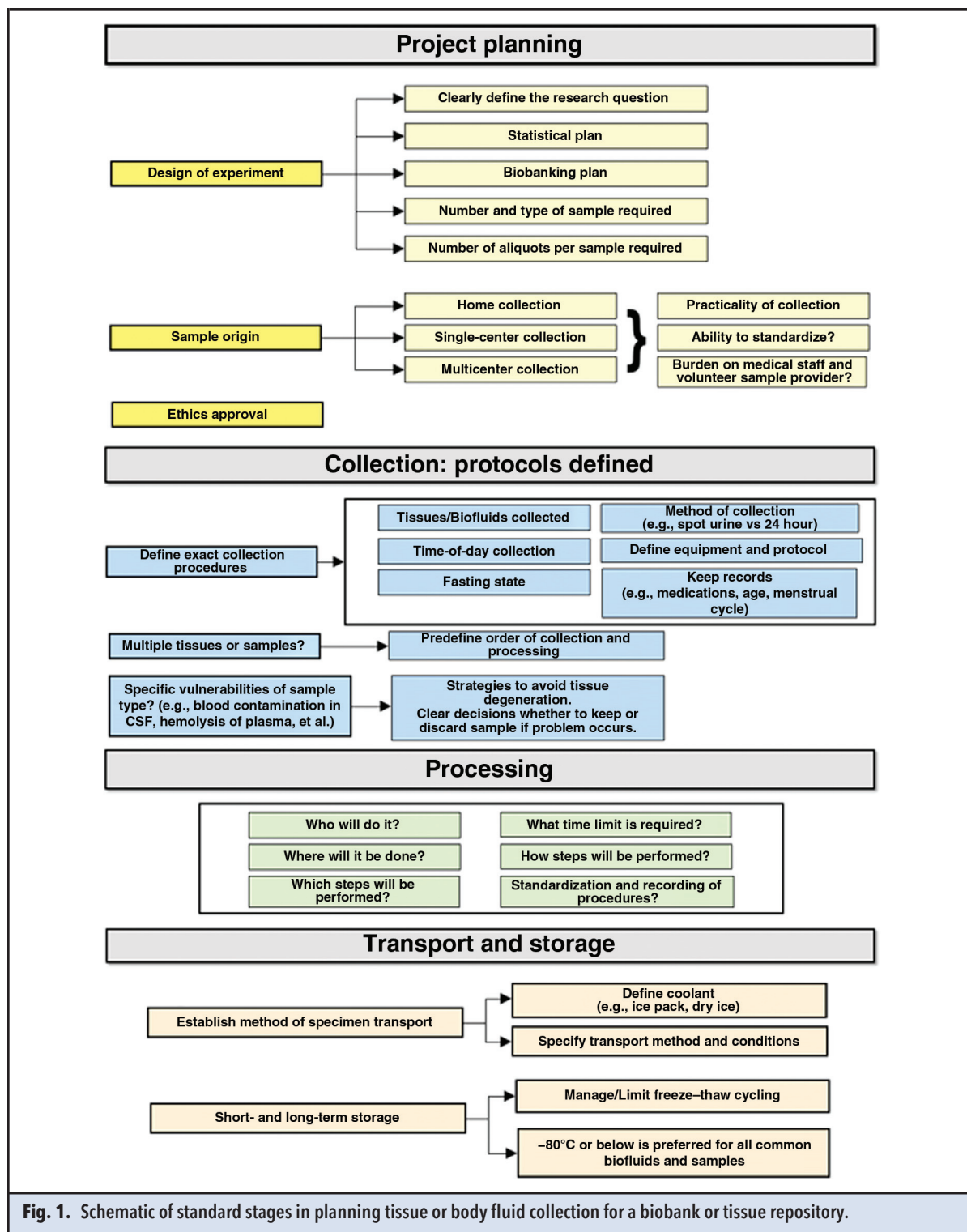
Project planning

Design of experiment
- Clearly define the research question
- Statistical plan
- Biobanking plan
- Number and type of sample required
- Number of aliquots per sample required

Sample origin
- Home collection
- Single-center collection
- Multicenter collection
}
- Practicality of collection
- Ability to standardize?
- Burden on medical staff and volunteer sample provider?

Ethics approval

Collection: protocols defined

Define exact collection procedures
- Tissues/Biofluids collected
- Time-of-day collection
- Fasting state
- Method of collection (e.g., spot urine vs 24 hour)
- Define equipment and protocol
- Keep records (e.g., medications, age, menstrual cycle)

Multiple tissues or samples? → Predefine order of collection and processing

Specific vulnerabilities of sample type? (e.g., blood contamination in CSF, hemolysis of plasma, et al.) → Strategies to avoid tissue degeneration. Clear decisions whether to keep or discard sample if problem occurs.

Processing

- Who will do it?
- Where will it be done?
- Which steps will be performed?
- What time limit is required?
- How steps will be performed?
- Standardization and recording of procedures?

Transport and storage

Establish method of specimen transport
- Define coolant (e.g., ice pack, dry ice)
- Specify transport method and conditions

Short- and long-term storage
- Manage/Limit freeze–thaw cycling
- −80°C or below is preferred for all common biofluids and samples

Fig. 1. Schematic of standard stages in planning tissue or body fluid collection for a biobank or tissue repository.

Table 1. Examples of protocols for tissue and biofluid collection for which validation is medium to high.

Tissue	Collection method	Time at 4 °C	Centrifugation before storage	Storage conditions	Biological variants discovered	Additional information	References
Serum/plasma	Venipuncture from arm; vein in inner elbow region is most appropriate and, if not possible, then from vein on the back of the hand (UK Biobank)	Blood samples to be stored at room temperature for up to 40 min maximum (HMGU)[a]; this includes clotting time for serum. This is followed by centrifugation at room temperature for 2750g for 10 min (HMGU) or 2500g for 10 min (UK Biobank). Samples are then stored at 4 °C and then on dry ice for transportation and subsequent processing (UK Biobank) or can be further processed and plasma or serum aliquoted into cryovials immediately (HMGU). Samples should be stored for longer periods after processing at −80 °C	2500g (HMGU) or 2750g (UK Biobank) for 10 min at room temperature	−80 °C	If stored at room temperature for considerable time, then instability of metabolites has been reported	Many external and internal factors have been shown to influence the metabolite composition of blood including fed/fasted status, age, gender, BMI[b], diet, ethnicity, etc.	(154, 155)
Urine	Collection in sterile container if possible at 4 °C (if not, ice pack will suffice for up to 8 h)	Minimize the time at 4 °C if possible; no longer than 8 h	Centrifugation is recognized as essential; protocols vary	−80 °C	Fasting samples are recommended; collection first thing in the morning is recommended (first void)	Dietary records or diet standardization is recommended, as diet is known to influence the urinary metabolome	(13, 80, 84)
Liver	Surgery (biopsy) or autopsy; in percutaneous biopsy, a gauge spring-loaded and cutting needle is passed under local anesthesia with an ultrasound guidance to collect the liver tissue samples	None Flash-frozen in liquid nitrogen quickly after collection	Most protocols do not use centrifugation step	−70 °C to −80 °C	Diet-induced variations in certain metabolites was reported	In post mortem tissue analyses, metabolomic data normalization should consider the time since death	(13, 80, 82, 156-158)
Muscle	Muscles were removed by surgery and trimmed of excess fat and connective tissue	None Flash-frozen in liquid nitrogen quickly after collection	Not reported	−80 °C	Age-related variations in certain metabolites were reported	Sterile instruments should be used to avoid contamination	(159-161)
Brain	Perfused tissue collection is preferred to nonperfused to reduce contamination of tissue with blood metabolites	None Flash-frozen in liquid nitrogen quickly after collection	Not reported	−80 °C		PBS or isotonic saline has been used as perfusion agent, but has not been validated for metabolomics	(64, 160, 162)

Table 1. Examples of protocols for tissue and biofluid collection for which validation is medium to high. *(Continued from page 1161)*

Tissue	Collection method	Time at 4 °C	Centrifugation before storage	Storage conditions	Biological variants discovered	Additional information	References
Colorectal tissues	Normally taken by pinch biopsies (~150–400 mg) from several locations of colon or rectum during colorectal resection	None Flash-frozen in liquid nitrogen quickly after collection	Not reported	–80 °C			(163–165)
Placenta	Placentas were normally sampled at 3 placental lobules; a sampling frame with intersecting lines was placed at the decidual surface, and chorionic plate was removed; the villous tissue was dissected into explants weighing approximately 10 mg per fragment	Flash frozen at –80 °C	Not standardly reported	–80 °C	Central placental lobes sampling was deemed the most representative and reproducible location to reflect optimal placental function	Placentas were collected within 20 min of delivery	(166, 167)
Lung	Surgical resection or core needle biopsy; tissue collection immediately after removal from thoracic cavity and flash-frozen in liquid N₂; both tumor and adjacent and distal nontumorous tissue should be sampled	None; flash-freeze in liquid nitrogen after blotting and rinsing in PBS Store at 77 K (–196 °C)	No	77 K (–196 °C)	Tissue at room temperature changes rapidly; freezing should be done in the OR[c] as soon as possible	Typically within 5 min of resection	(75, 168, 169)

[a] Helmholtz Zentrum Muenchen fuer Gesundheit und Umwelt.
[b] Body mass index.
[c] Operating room.

Table 2. Examples of protocols for tissue and biofluid collection for which further validation is strongly recommended.

Tissue	Collection method examples	Range of temperatures before processing	Centrifugation ranges before storage	Storage condition ranges	Reported consequences	Biological variants discovered	Additional information	References
Saliva	Stimulated and nonstimulated methods exist; nonstimulated potentially better	4 °C for between <30 min and <24 h; shorter periods likely to be preferable but not fully validated	Between 1 to 20 min at 1000–2600g; before freezing may be preferable but not fully validated	−20 to −80 °C		Large interindividual and intraindividual variation not reduced by standardizing diet for 24 h	Prerinsing and abstinence from oral products, food and smoking may be important but unvalidated	(12, 13, 110, 111, 117, 120)
CSF	Lumbar puncture	Centrifuge as quickly as possible after collection; freeze within 1 h	2000g for 10 min at 4 °C before freezing	−80 °C	Cells in the sample will degrade it at room temperature, so centrifuging is crucial step; storage at temperatures lower than −80 °C will change the pH	Age, diet, diurnal and seasonal variations in certain metabolites reported	Discard first 2 mL of CSF	(9, 122–124)
Sweat	Induction via chemical stimulation with pilocarpine most common (also heat or exercise); collection normally with purpose designed instrument or absorbent material	Avoiding evaporation of sweat before volume accurately calculated is most important consideration; working temperatures following collection are often not recorded	Not standardly used but has been used to prevent epithelial cell contamination	Normally −80 °C	Varying interindividual sweat rates	pH can vary widely so can affect analysis of acid and bases; standard range of biological variants are seen in sweat	Skin should be clean and free from topical products before measuring	(170–176)
Milk	Self-expression or breast pump, use of fore or hind milk should be specifically stated	Freeze as soon as possible after collection because of risk of degradation	Most protocols do not use centrifugation	Between −18 °C (home freezer) and −80 °C; −80 °C should be used for medium- and long-term storage	Freezing agglutinates micelles and reduces risk of insoluble particles in solvent extraction	Lactation stage heavily influences profile; mothers with premature babies have different milk profiles than term mothers at the same lactation stage; "secretor" and "non secretors" have different profiles in oligosaccharides	When milk is taken, record which breast, day of lactation, hours after last feed, and what part of milk is used	(177–181)
Lacrimal	Rubbing inferior meniscus and collecting lacrimal secretions using a micro Pasteur pipette/Schirmer strips (free catch reported but not yet used for metabolomics)	Freeze as soon as possible after collection because of risk of degradation	Not standardly reported	−80 °C or colder	Tears are laden with enzymes that may degrade the sample			(137, 182)
Endometrial/ peritoneal fluids	Normally taken transvaginally using an embryo transfer catheter (endometrial fluid) or at laparoscopy using a syringe or suction device (peritoneal fluid)	4 °C or on ice until processed (which should occur as soon as possible)	Yes, but values are not standardized	−80 °C or colder	Molecular profile of peritoneal lavage fluid may change dependent on method used	Peritoneal fluid increases in follicular phase of the menstrual cycle	Record day of menstrual cycle	(115)

Table 2. Examples of protocols for tissue and biofluid collection for which further validation is strongly recommended. *(Continued from page 1163)*

Tissue	Collection method examples	Range of temperatures before processing	Centrifugation ranges before storage	Storage condition ranges	Reported consequences	Biological variants discovered	Additional information	References
Microdialysate	Microdialysis catheter has a semipermeable membrane tip that can be placed in the tissue of interest; an aqueous fluid is infused through the tip, metabolites in the extracellular membrane passively diffuse over the membrane and up through a separate channel in the catheter where they can be subsequently collected for analysis	Often not recorded, but ideally should be collected on ice given the long collection periods that often occur	Not validated	Not validated	Having a low flow rate of the microdialysis fluid enables better equilibration between tissue and microdialysate		Focal technique, which may be of interest to researchers studying localized reactions; Not used much for metabolomics	*(143, 144, 183, 184)*
Breath and volatiles	Exhaled breath condensate collected by use of a precooled closed condenser design system made from inert materials with a filter to remove environmental contaminants	Collect on dry ice and immediately freeze to preserve volatiles; freeze-drying has been proposed to concentrate samples but not yet validated; note temperature of collection (and ambient temperature, as this creates the temperature gradient); use nose clips and saliva traps	Not standard, has not been validated for metabolomics	Coldest available (−80 °C or lower)	Food and drink significantly affect measurements	Leukotrienes degrade within a few months of storage	Degassing to remove CO_2 may reduce variability in analysis because of pH but is not validated; other methods of collecting breath and volatiles also exist	*(147, 148)*
Heart	Cardiac biopsies (∼100 mg) collected from the epicardial surface; some methods recommend rinsing with PBS after removal	Frozen in liquid nitrogen	Not reported	−80 °C or colder		Gender-specific changes in metabolite profiles were observed		*(185–187)*
Pancreas	Biopsy or autopsy (directly excised using scissors)	Flash-frozen in liquid nitrogen	Not reported	−80 °C				*(188, 189)*
Eyes	Enucleation was performed and ocular tissues (cornea, lens, vitreous, and retina) were carefully dissected under microscopic guidance; however, some performed retinal dissection in ice-cold PBS (in mmol/L: 137 NaCl, 2.7 KCl, 10 Na_2HPO_4, 1.8 KH_2PO_4, pH 7.4)	All dissections were performed on a cold plate to maintain a temperature of approximately 4 °C, and dissection time was between 2 and 5 min; after that, the samples were flash-frozen in liquid nitrogen	Not reported	−80 °C	Waste products of metabolism from cornea and lens are secreted into the aqueous humor and vitreous directly or move into the vitreous indirectly via diffusion, which can cause the similarities between the metabolites found in these 3 tissues		Enucleation should be performed as soon as possible (between 1 and 20 min) after the death	*(190, 191)*

Table 2. Examples of protocols for tissue and biofluid collection for which further validation is strongly recommended. *(Continued from page 1164)*

Tissue	Collection method examples	Range of temperatures before processing	Centrifugation ranges before storage	Storage condition ranges	Reported consequences	Biological variants discovered	Additional information	References
Endometrium	Laparoscopic surgery using endometrial sampling device or Pipelle catheter (a plastic tube withdrawn to create suction, rotated and moved outward to collect small pieces of endometrial tissues) are commonly used methods	Frozen in liquid nitrogen	Not reported	−80 °C	Extraction and storage should occur within 15 min to minimize enzymatic degradation		Menstrual phase should be determined from an endometrium sample	*(192, 193)*
Spleen	Biopsy or autopsy (directly excised using scissors)	Flash-frozen in liquid nitrogen	Not reported	−80 °C				*(194–196)*
Kidney	Biopsy (skin is cleaned using alcohol swab; incision is made to cut open skin and muscle to expose the kidney to collect target region)	Flash-frozen in liquid nitrogen	Not reported	−80 °C	Well-known heterogeneity for different regions		Body temperature should be monitored and controlled	*(65)*
Testes	Biopsies or surgical explorations	Flash-frozen in liquid nitrogen	Not reported	−80 °C				*(197, 198)*
Intestine	Normally removed surgically, flushed with PBS	Flash-frozen in liquid nitrogen	Most of the protocols do not use centrifugation step	−80 °C				*(156)*
Adipose	Biopsy (a gauge needle is inserted into the subcutaneous fat, and 5 mL of 0.9% sodium chloride is injected; a syringe with locking piston is used to create a vacuum and a back-and-forth motion used to harvest the tissue; once the tissue has been aspirated, the needle is withdrawn and the piston removed)	Flash-frozen in liquid nitrogen within 45 min	Not reported	−80 °C			Tissues were collected after an overnight fast	*(199)*
Esophagus	(a) Endoscopically during staging laparoscopy under a general anesthetic, (b) during endoscopic ultrasound staging under sedation, or (c) surgical specimens immediately after retrieval during esophagectomy	Immediately frozen in liquid nitrogen after dissection	Not reported	−80 °C			Tissues were collected after an overnight fast	*(200)*

Table 2. Examples of protocols for tissue and biofluid collection for which further validation is strongly recommended. *(Continued from page 1165)*

Tissue	Collection method examples	Range of temperatures before processing	Centrifugation ranges before storage	Storage condition ranges	Reported consequences	Biological variants discovered	Additional information	References
Dried blood spots	Do not use the tip of center of the finger; do not use the second (index) and fifth (small) finger; avoid the side of the finger, and use the top of the finger and a lancet to pierce the finger (perpendicular to the fingerprint ridges); press the finger and wipe away the first drop of blood; hold the finger over the card, and when a drop is present, then place drop onto card but do not press finger onto card; collect 3 drops; allow blood spot to dry for a minimum of 3 h at room temperature, and do not speed up drying with additional heat	Room temperature appears appropriate for short-term storage but further evidence/stability studies required	Not required	Long-term stability studies not currently published for nontargeted metabolomics; stability of specific analytes has been shown for room temperature or reduced temperatures for up to years	Long-term storage at room temperature can be performed; storage at reduced temperatures recommended if stored in a sealed bag with a desiccant	Many external and internal factors have been shown to influence the metabolite composition of blood, including fed/fasted status, age, gender, BMI[a], diet, ethnicity, etc.	Not applicable	*(201)*

[a] Body mass index.

Table 3. Summary of preanalytical methods for the collection and storage of fecal samples.

Source	Population	Sampling method	Sampling temperature	Preservation temperature	Processing method
¹H-NMR					
Marchesi et al., 2007 (101)	CD[a] and UC[b]	Stool	Frozen at −80 °C immediately	−80 °C	Fecal water derived from weighed sample of bulk stool
Jacobs et al., 2008 (103)	Healthy	Stool	Homogenized and frozen immediately	−20 °C	Lyophilized
Saric et al., 2007 (102)	Healthy	Stool	NA[c]	NA	Fecal water
Bezabeh et al., 2009 (202)	CC[d]	Stool	−20 °C for 24-48 h; transported on ice	−70 °C	Fecal water derived from frozen, homogenized bulk stool
Monleón et al., 2009 (100)	CC	Stool	−20 °C for 1 h	−80 °C	5 g of sample extracted from frozen bulk stool and homogenized to produce fecal water
Le Gall et al., 2011 (203)	IBS[e] and UC	Stool	Ice for 2 h	−70 °C	Fecal water
Bjerrum et al., 2014 (204)	Active or inactive human UC and CD subjects	Bulk stool	Ice for <3 h	−80 °C	Fecal water derived from weighed sample of bulk stool
Gratton et al., 2016 (97)	Healthy–optimized sample collection and management	Stool	Ice for 1 h; extracted within 24 h of collection	−20 °C or more; avoid free-thaw cycles	Fecal water
GC-MS					
Gao et al., 2009 (205)	Healthy	Stool	Aliquoted and frozen at −80 °C after defecation	−80 °C	Fecal water derived from homogenized 5-8 g samples of stool
Gao et al., 2010 (206)	Healthy	Stool	Processed immediately after collection	−80 °C	Fecal water from homogenized bulk stool
Ponnusamy et al., 2011 (207)	IBS and non-IBS	Stool	Frozen immediately at −20 °C	−80 °C	Fecal water derived from homogenized 0.1-g stool isolates
Poroyko et al., 2011 (208)	Neonatal ICU[f] infants	Stool	Frozen immediately at −80 °C	−80 °C	Lyophilized just before analysis
Ng et al., 2012 (209)	*Cryptosporidium* positive and negative	Stool	Stored at 4 °C	−80 °C	Fecal water derived from 250 mg (wet weight) lyophilized feces
Weir et al., 2013 (210)	Healthy and CC	Stool	Transported to lab within 24 h	−20 °C	Bulk stool homogenized; 3 samples taken with swabs; samples lyophilized
Phua et al., 2013 (105)	Healthy	Stool	Feces pooled from 2 males, 2 females, and lyophilized	NA	Lyophilized
Goedert et al., 2014 (211)	Healthy and CC	Stool	Frozen at home on dry ice	−40 °C	100 mg of lyophilized feces
Hintze et al., 2014 (212)	Healthy	Stool	Frozen immediately at −80 °C	−80 °C	Dried under vacuum
Ahmed et al., 2016 (213)	UC and CD	Stool volatiles	Submitted with 6 h of collection	−20 °C	Headspace gases

Storage and preparation parameters

Table 3. Summary of preanalytical methods for the collection and storage of fecal samples. *(Continued from page 1167)*

Source	Population	Sampling method	Storage and preparation parameters		
			Sampling temperature	Preservation temperature	Processing method
LC-MS					
Cao et al., 2011 (214)	LC[g] with hepatocellular carcinoma and healthy	Stool	Aliquoted and frozen immediately after defecation	−80 °C	Fecal water
Girlanda et al., 2012 (215)	Intestinal transplant: rejection vs nonrejection	Ileostomy fluid or stool	Stored at 4 °C for <12 h immediately after collection	−80 °C	Fecal water
Huang et al., 2013 (216)	LC and healthy	Stool	Aliquoted and frozen immediately after defecation	−80 °C	Fecal water extracted taking a weighed sample of aliquoted, thawed stool
Loftfield et al., 2016 (99)	Healthy	Stool	Freeze 1- to 2-g aliquots within 30 min of bulk stool collection at −80 °C	−80 °C	Compare frozen (no solution) with 95% ethanol, FOBT[h], FIT[i]
CE-MS[j]					
Tomitsuka et al., 2017 (217)	Healthy individuals on low and normal protein diets	Stool	Transported to the lab and cooled (about 7 h) in Anaero Packs (Mitsubishi Gas Chemical); 2.0 g of total then frozen in liquid nitrogen dry vapor, transported further at −150 °C and then stored at −80 °C before processing	−80 °C	Fecal water extracted in PBS using 0.5 mg of feces in 1 mL of PBS; centrifuged and filtered

[a] Crohn disease.
[b] Ulcerative colitis.
[c] Not reported.
[d] Colorectal cancer.
[e] Irritable bowel syndrome.
[f] Intensive care unit.
[g] Liver cirrhosis.
[h] Fecal occult blood test.
[i] Fecal immunochemical test.
[j] Capillary electrophoresis-mass spectrometry.

ered, resulting in a much higher probability of false-negative findings (type II error). Similarly, systematic differences in sample collection (e.g., disease samples collected using a different protocol than for control samples) result in biased statistical analysis, often with 100% confounding factors, which increases the probability of both false-positive and false-negative results (type I and II error). These issues have been discussed at great length previously (25), but it is important to note that compounding multiple errors, because of collection variance at multiple stages of the complete work flow, will rapidly inflate the probability of false discovery. As such, detailed consideration should be given to validating and optimizing each step of the work flow and ensuring consistent collection and processing of samples through standard operating procedures and staff training.

Preanalytical Processing and Biobanking of Serum, Plasma, and Dried Blood Spots

Blood-based biofluids and urine are the most frequently collected human samples in metabolomic and epidemiological studies. These biofluids provide a "global" view of metabolism operating in multiple different tissues and organs and are relatively easy to collect. No single best practice protocol is currently established, and samples are typically collected according to standard blood collection protocols (Table 1). However, as discussed below, there are several considerations when assessing the applicability of blood collection and processing for samples already collected or for samples to be collected in the future. Consistency is important whichever collection strategy is used.

Large biobank repositories containing blood products (serum and plasma) are available for researchers to access samples (e.g., the UK Biobank contains serum and plasma samples for 500 000 individuals) (26). Trained phlebotomists normally collect blood, although the alternative option of dried blood spot (DBS)[11] collection is available. DBS offers an opportunity for collection in environments away from the clinic, without the presence of trained staff and without the time-related requirement of sample centrifugation and processing. A lancet is used to provide a finger prick to produce a small volume of blood (up to 25 μL typically), which is physically spotted onto filter paper-based collection cards (e.g., GE Healthcare's Whatman 903 cards) (27) and then allowed to dry before storage (28, 29). This option allows for collection of samples across a time series by the subject, and samples can be sent via postal services for storage in clinical bio-

banks (e.g., a targeted vitamin D assay is operated in this way) (30). Most applications focus on a targeted set of metabolites including amino acids and acylcarnitines for inborn errors of metabolism testing (31, 32) or drugs and their metabolites in drug metabolism and pharmacokinetic studies (33). The hematocrit in the blood spot and sample stability (34, 35) are important factors to consider. Although the use of DBS for untargeted metabolomics applications is not frequently used (36), there is a growing interest in its potential use because DBS offers some advantages over plasma and serum and removes several preanalytical factors discussed below. However, 1 major consideration is the stability of DBS at different drying times, drying and storage temperatures, and storage times (35, 36). Further investigations of the stability of different metabolite classes or individual key metabolites are required before epidemiological studies using DBS can be performed.

Collection of Blood Products

Plasma and serum can be considered as 2 different biofluids both originating from blood. Plasma is collected using anticoagulants and, thus, preserves most clotting factors, whereas serum is produced using a natural blood clotting process. The question of whether serum or plasma should be collected is important because the production of serum involves coagulation, whereas plasma does not. The time and temperature allowed for coagulation are important preanalytical factors to consider and should be standardized for sample processing. Special care should be taken during drawing and handling of samples to avoid hemolysis. The release of hemoglobin and other intracellular components from erythrocytes can markedly alter plasma/serum metabolomic profiles (37, 38); thus, hemolyzed blood samples should be avoided in metabolomics studies. Studies comparing serum and plasma from the same individuals have described only small qualitative and quantitative differences for the metabolite composition of the 2 biofluids, and this variability is less when comparing with the biological variability observed between different persons (39–41). For example, higher concentrations of some metabolites have been reported for serum compared with plasma collected from the same individuals; in 1 study, 9 metabolites were reported to have >20% greater concentrations in serum than in plasma including amino acids (glycine, serine, phenylalanine, and arginine), lysoglycerophospholipids (C16:0, C17:0, C18:0, and C18:1), and glycerophosphatdylcholines (C38:1) (39). Therefore, there is no clear conclusion on which biofluid to collect, although the same biofluid should be used in any single study. Plasma may have some advantages over serum, as a more standardized collection and extraction procedure

can be used without concern regarding how the clotting procedure may influence the metabolome *(42)*.

The collection of blood and its processing should be performed according to standard protocols (Table 1). Preanalytical factors are important for blood, which is metabolically active, primarily through cellular metabolism (especially erythrocytes), to ensure that the postprocessed sample is a qualitative and quantitative representation of the sample at the time of collection *(43)*.

Time of Sample Collection

The time of day a blood sample is collected can influence the quantitative composition of serum and plasma samples as shown by several diurnal studies *(11, 44, 45)*. The time of day and season were not significant sources of variation in 1 study, but it is recognized that this finding is not conclusive for all studies, and a standardized collection within the same time of day and in either fasted or fed status is recommended *(46)*.

Type of Plasma Collection Tube

Three standard types of anticoagulants can be applied for collection of plasma samples: lithium (or sodium) heparin, sodium citrate, and potassium ethylenediaminetetraacetic acid (EDTA) *(30)*. Citrate and EDTA are low molecular weight chemicals and can interfere in the detection of metabolites in untargeted metabolomics studies and will also be detected by most analytical platforms. In addition, 2 further disadvantages of citrate include that it is an endogenous metabolite and that it can alter the pH of the sample and, therefore, the extraction conditions *(47)*. Heparin is not detected in a typical untargeted metabolomics assay, which is advantageous, although the introduction of lithium into the sample can result in unwanted lithium adducts in mass spectrometric analyses. EDTA-based samples are also used frequently and may be more advantageous if concurrent proteomics is planned with the same sample.

Type of Serum Collection Tube

There are 2 types of serum collection tubes: gel-free and gel-containing (polymeric) tubes *(48)*. The quality of these tubes is important, and the use of gel-free tubes removes the possibility of sample contamination from the gel (polymer). Lopez et al. assessed polymeric and nonpolymeric tubes for serum and EDTA plasma processing and showed no differences in the metabolic profile for plasma EDTA tubes but did show differences between polymeric and nonpolymeric tubes for serum, although no preferred tube was indicated *(49)*.

Inversion of Plasma Tubes

Tubes are inverted gently several times to allow mixing of anticoagulants with whole blood, and a lack of standardization of this process can be expected to influence the qualitative and quantitative composition of the processed plasma sample.

Processing Location, Temperature, and Time Intervals

Whole blood can be processed at the site of sample collection or at a central processing facility *(50, 51)*. Studies have investigated whether transport to a central processing facility can be undertaken rather than rapid processing and concluded that, if samples were stored cold (4 °C), delayed processing had only minor influences on the metabolic profile, and the differences observed related to processing were much smaller than intersubject differences *(21, 52)*. Processing should be undertaken before freezing the samples because freezing whole erythrocytes will result in their lysis and substantial contamination of the plasma metabolome.

The associated temperature for storage and the time between whole blood collection and centrifugation are important preanalytical factors. Whole blood stored at room temperature is much more metabolically active than that stored on ice at approximately 4 °C, which will greatly affect the quantitative metabolic profile. We recommend a short processing time (<30 min) if storage is at room temperature, but storage at 4 °C on ice or in a refrigerator for longer periods is accepted by some large biobanks. Again, the variability introduced by different storage times and temperatures is low compared with the biological variability observed when studying the human population *(8, 21, 52–56)*. Two separate studies have recommended processing within 3 h at 4 °C whenever feasible *(8, 53)*. Jain et al. *(55)* have demonstrated metabolic changes measured up to 20 h before centrifugation, with metabolites related to erythrocyte metabolism showing the greatest change, and with lactate and arginine/ornithine being potential markers of instability. Trezzi et al. *(8)* have established a novel blood sample quality control marker, the LacaScore, based on the ascorbic acid/lactate ratio in plasma.

Centrifugation time and temperature must be consistent according to a standard operating procedure (Table 1). Some metabolites are unstable even under acceptable processing conditions, including cysteine and cystine *(54)*.

Long-Term Storage Temperature

Until recently, plasma and serum were considered metabolically inactive after separation from cells in whole

blood. Quantitative changes in some metabolites can occur even in samples stored at −80 °C, often displaying nonlinear patterns of concentration change (57). Therefore, we recommend that samples always be stored frozen, preferably in liquid nitrogen or at −80 °C when liquid nitrogen is not available and analyzed within the shortest time window possible after collection. We recommend storing samples from an individual in multiple 0.5- or 1.0-mL aliquots. This minimizes any need for multiple, potentially damaging, freeze–thaw cycles should an individual's sample require multiple analyses (56). The number of temperature change cycles, e.g., because of the freezer door being open, is also likely to be important for all tissue types, although thus far little investigation has been done on this phenomenon (58).

Sample Stability

Although inappropriate thawing of samples and multiple freeze–thaw cycles are likely not appropriate, limited research has been reported to assess this process. Pizzaro et al. have reported that the thawing temperature was important and recommended thawing small aliquots (0.25 mL) on ice for lipids and that ultrasound thawing improved sensitivity and detection for lipid studies (59). Breier et al. showed that 2 freeze–thaw cycles did not influence the metabolic profiles in a targeted assay (21), but when possible, individual laboratories should conduct their own freeze–thaw investigations on metabolites of interest. Where freeze–thawing cannot be avoided, the number of freeze–thaw cycles should be recorded and standardized among samples in the same study to avoid bias and enable it to be included as a confounding factor when analyzing the data.

RECOMMENDATIONS
Blood products are probably the best studied and validated biofluids for metabolomics. Therefore, we recommend that the selection of an appropriate protocol is based on access to validation data demonstrating the robustness of the protocol.

Preanalytical Processing and Biobanking of Tissues

A detailed metabolic phenotypic analysis of tissues reflects their physiological and pathological status (60). However, obtaining systematic and reproducible results in tissue samples is challenging because of the complex nature of the samples and diversity of the metabolites. Thus, establishing work flow pipelines and standardized protocols for tissue metabolomics is of utmost importance, especially focusing on the preanalytical steps, which can introduce huge variability in the subsequent analyses (61–64). The evidence base for different tissue types is variable. A

summary of protocols for tissues with medium to well-defined evidence-based protocols is outlined in Table 1, whereas tissues for which substantially more validation research is needed are featured in Table 2.

Collection of Tissues

Want et al. (65) recommended that the timing of the tissue sample collection for controls and cases be randomized. When compared with collection of biofluids or blood, which can be collected easily and noninvasively, additional care should be taken for tissue samples. Tissues are normally collected with anesthesia or at autopsy, which may pose several problems. To obtain reliable metabolite profiles, sampling should occur as quickly as possible, which can create challenges in an operating theater (43). To measure extremely labile metabolites as accurately as possible, subsequent immediate flash-freezing on collection is required to avoid further metabolism in the samples (66). However, in an operating theater, there may be safety issues with having liquid nitrogen present. By contrast, placing a warm tissue directly into a −80 °C freezer may enable the metabolic profile to change in the time it takes for the sample to freeze and, thus, is not optimum. To avoid contamination, as much blood as possible should be removed from the tissue samples (67). Because most tissues are heterogeneous, for repeatable results the location of the tissue sampling should be consistent throughout the same experiment to avoid region-specific metabolite variance if there is a limitation in obtaining the whole tissue for metabolomics analyses (65). Usually needle (e.g., organs) (68, 69) or punch (e.g., skin) (70) biopsies are carried out to collect the human tissues for metabolomics analyses, and care should be taken to avoid unnecessary biopsy-associated complications in the patients (71).

Important spatial information can be obtained from tissue sample metabolite analyses. Latest state-of-the-art technologies like mass spectrometry-based imaging analysis using, for example, MALDI-TOF MS, facilitates "spatial metabolomics" (72, 73). For tumor sample analysis, additional care should be taken about the location of sample collection (e.g., oxygenated vs necrotic areas) (65) and collection of "normal" tissue as a control (74, 75). Hence, careful and detailed experimental design is required considering what is the best representative tissue sample in terms of location, sample amount, and the required number of biological replicates for better statistical power in data analyses.

Storage of Tissues

As for blood, tissue samples should be stored at −80 °C or lower for long-term storage for future metabolomics analyses. Sectioning large tissue samples into smaller

pieces may facilitate quick freezing and avoid future freeze–thaw cycles, although consideration should be given to the likely metabolite changes that may occur dependent on the sectioning technique and resulting time delay. However, considering that tissues are heterogeneous samples (e.g., metabolomic alterations during acute kidney injury are different in kidney cortex and kidney medulla), another possibility is using a tissue homogenizer or a clean uncontaminated mortar and pestle with liquid nitrogen and subaliquoting by weight to obtain homogeneous and representative samples *(76–78)*. Unlike other sample types, there may be other requirements for their use, such as histology, which may influence which protocols can be used for processing for storage. There are usually only limited amounts of tissue samples available, especially in the case of human biopsies. Thus, care should be taken while collecting for subsequent aliquoting and storing at appropriate conditions, with consideration given to avoid contamination when slicing or homogenizing.

RECOMMENDATIONS
We recommend that tissue-specific sampling protocols be used. In general, tissue samples should be collected as quickly as possible, flash frozen in liquid nitrogen, and stored at −80 °C or lower for long-term storage. Freeze–thaw cycles should be minimized as much as possible, and subaliquoting the tissue samples while collecting will reduce the future freeze–thaw cycles. For repeatable results, the location of the tissue sampling should be consistent throughout the same experiment to avoid region-specific variance.

Preanalytical Processing and Biobanking of Urine

In recent years, metabolomic studies using urine have clearly demonstrated its usefulness in a range of disciplines. Furthermore, urine is easy to collect and is abundant with metabolites that reflect food intake, environmental exposures, and alterations in biochemical pathways. However, to ensure that the results obtained are meaningful, it is imperative that attention is given to sample collection conditions, storage conditions, and postcollection treatment. A summary of protocols can be found in Table 1.

Collection of Urine

Urine samples can be collected as either a single midstream collection at a specific time (spot urine samples) or the total urine output in a period of 24 h (24-h samples). In both cases, it is important to prevent bacterial growth and ensure stability of the metabolites. It is now accepted that collected human urine is not sterile because of contamination by bacteria in the urethra *(79)*. Indeed, bac-

terial growth in collected urine can be rapid and can modify metabolite composition. A recent study designed to examine the impact of sample collection conditions on the metabolite composition demonstrated that addition of preservative prevented bacterial growth but did not avoid metabolite degradation *(80)*, whereas storage at 4 °C inhibited bacterial growth and metabolite degradation. This has important consequences for collection of 24-h urine samples; to preserve the integrity of the sample, the results from this study support maintaining samples at 4 °C during the collection period.

Depending on the research question, either spot urine or 24-h urine samples can be collected. A research question may be more suited to the use of one over the other. Participants' state of fasting is important for spot urine collection. Food intake has a substantial impact on the urinary metabolic profile *(13)*, which should be considered in the design of collection protocols.

An advantage of using urine is the possibility of home sampling, which facilitates interventional study designs. However, special care must be taken regarding the use of inadequate urine collection containers and improper conditions of storage and transportation to repositories *(81)*.

Processing Location, Temperature, and Time Intervals

A recent study by Rotter et al. *(82)* used a targeted approach to measure the stability of 63 metabolites under different storage conditions in the short term—a period that reflects the sample collection period. More specifically, the study investigated storage at −80 °C, −20 °C, 4 °C, 9 °C (cool pack), and room temperature for 0, 2, 8, and 24 h. Interestingly, about 90% of the targeted metabolites were not significantly altered by any of the conditions when compared with the samples immediately frozen at −80 °C. However, storage in a cool pack and at room temperature resulted in changes in certain metabolites. Therefore, their recommendation was not to transport or store samples for >8 h in a cool pack or at room temperature. By contrast, the Da Vinci biobank recommends storing urine for nuclear magnetic resonance (NMR) for no more than 2 h at 4 °C and centrifuging before freezing to avoid cell lysis *(83)*.

Processing of collected urine is equally important. Bernini et al. *(84)* examined this step and concluded that a mild centrifugation and filtration step is necessary to ensure removal of cellular components. Care is required with filtration because there is the potential for loss of metabolites. As a result, many studies process urine samples using centrifugation only. Typical centrifugation conditions include centrifugation at 2000g for 10 min (see Table 1).

Storage of Urine

Once collected, a decision on the long-term storage conditions for the samples is necessary. Leparre et al. recently examined the long-term storage of urine samples using both targeted LC-MS metabolomics and a global profiling approach and found that storage at −20 °C for short periods (up to 20 days) was sufficient but that storage at −80 °C was recommended for longer periods to ensure the stability of sensitive metabolites *(85)*. This is also supported by previous literature predominantly performed using NMR as the analytical technique. Earlier studies using NMR demonstrated that at storage conditions below −20 °C for 26 weeks there was limited impact on metabolite profile *(86)*. Shorter-term studies also demonstrate stability of metabolites when stored at −80 °C and changes in metabolite profiles when storage was performed at room temperature *(80, 84, 87)*.

Sample Stability

Several studies have examined the effects of freeze–thaw cycles on metabolite concentrations in urine samples. Earlier studies investigating the impact of freeze–thaw cycles focused on metabolite profiles and assessed the statistical impact using principal component analysis. Gika et al. examined the metabolite profile and reported no major global changes after 9 freeze–thaw cycles *(88)*. However, using targeted analysis, Saude and Sykes reported an intermediate amount of change in metabolite concentrations after 8 freeze–thaw cycles *(89)*. More recently, Rotter et al. demonstrated that 2 freeze–thaw cycles had no impact on metabolite concentrations *(82)*. However, after 3 cycles, significant effects were identified, leading to the recommendation to limit the number of freeze–thaw cycles.

RECOMMENDATIONS
The overall evidence supports the following recommendations: storage of urine samples chilled during collection (4 °C if possible), centrifugation as soon as possible, and long-term storage at −80 °C. Addition of a preservative is not necessary if samples can be maintained at 4 °C. When frozen, the number of freeze–thaw cycles should be limited to 2.

Preanalytical Processing and Biobanking of Feces

Sample acquisition, sample processing, and sources of experimental variability in serum, plasma, and urine metabolomics studies have been characterized in greater depth *(90–92)* than in the field of fecal metabolomics. Substantial progress has been made in nucleic acid-based metagenomic analysis of fecal microbial composition and diversity. The literature on sample acquisition, processing, and management for fecal metabolic phenotyping is less well developed (Table 3). Although LC-MS and GC-MS are the most widely used analytical platforms in metabolomics, the use of ^{1}H NMR spectroscopy has been the most common tool used in fecal metabolic phenotyping *(93, 94)*. The present discussion addresses critical elements to be considered in sample acquisition, a minimal set of recommended elements of sample acquisition, and a recognition that much work is needed to establish best practices for this specimen type.

Feces are considered a noninvasive proxy for the study of the intestinal microbiome and metabolome. As a specimen, feces present several analytical challenges because solid waste is a heterogeneous complex of dietary precursor molecules, undigested material, microbes, microbial fragments, microbial metabolites, and host cellular and molecular elements. In this context, the gut metabolome is a representation of host metabolism, microbial metabolism, host–microbial cometabolism, and environment *(95)*. For these reasons, removal of solid particles, dead bacteria, and undigested matter from fecal specimens is frequently considered *(96)*. This contrasts with urine and plasma, which generally require no extraction and to which the addition of buffer solutions is sufficient.

Collection of Feces

To adequately reflect the associated molecular diversity, the manner of feces collection is of considerable importance. There are 2 basic specimen considerations. The most simple and scalable method (for large studies) is to obtain a fecal specimen by paper tissue or swab (spot sampling). The alternative method is the use of whole bulk feces, which can be aliquoted for specific analytical uses. Although the fecal metabolome literature using the swab technique is limited, the stool microbial metagenome literature is instructive. Several technologies exist to extract spot samples for microbial metagenome analysis. However, to date, the fecal metabolome literature predominantly reports on bulk stool as the primary specimen. A recent NMR-based fecal metabolome study examined stool homogeneity, obtaining specimens from 4 locations (e.g., top, edge, middle, and bottom) of the bulk stool specimens. Principal component analysis revealed that the crude fecal samples collected from different positions were widely distributed in the metabolic space, and orthogonal partial least-squares discriminant analysis further showed that the sample composition between topographical positions and whole sample homogenate was significantly different *(97)*. This contrasts with microbial species composition, which has been observed to be more homogeneous across bulk stool *(97)*. From the evidence to date, a balance must be struck between

ease of sample collection and the heterogeneity of bulk feces. When considering either spot or bulk sampling method, issues of the topographical homogeneity/heterogeneity of the fecal specimen should be considered.

Timing related to collection of stool is generally random unless modifiers of stool frequency and consistency (e.g., laxatives) are used. When such modifiers are used, the method used and its influence on the metabolome should be carefully noted and considered. Timing of food intake should also be considered. Stool microbiome studies have generally been conducted, using overnight fasts, unless specific dietary questions are being addressed in the study. A recent study showed that a given dietary substrate load (e.g., banana vs cookie) may evoke substantially different responses between individuals (e.g., serum glucose) and that these differences may, in part, be driven by the gut microbial community structure *(98)*. Thus, dietary intake should be carefully controlled or monitored, depending on the research question. Samples collected with no additives are suitable for untargeted metabolomics analysis when frozen shortly after collection, i.e., storage at −80 °C within approximately 30 min after collection *(99)*. This is considered the gold standard in fecal metabolome analysis. Recently, Loftfield et al. collected fecal samples from 18 volunteers, using 4 methods: no solution, 95% ethanol, fecal occult blood test (FOBT) cards, and fecal immunochemical test (FIT). One set of samples was frozen after collection (day 0) and for 95% ethanol, FOBT, and FIT. A second set was frozen after 96 h at room temperature. Using untargeted metabolomics, stability was examined after 96 h at room temperature for 95% ethanol, FOBT, and FIT, along with concordance of metabolite measures with frozen day 0 samples without solution *(99)*. Metabolite concordance with the gold standard was higher for 95% ethanol (median $r_s = 0.82$), followed by FOBT (median $r_s = 0.70$) and FIT (median $r_s = 0.40$).

There is a general question regarding which type of preprocessed sample is optimum for fecal metabolome analysis. Presently, there are 3 primary sample types being explored, which include crude fecal samples *(100)*, fecal water extracts *(97, 100–103)*, and lyophilized feces *(103, 104)*. Each may yield a slightly different metabolite profile. For instance, Phua et al. compared lyophilized human feces vs fecal water using a GC-TOF MS method. Lyophilized feces demonstrated a larger number of chromatographic peaks (704 vs 664), reflecting more comprehensive metabolic coverage. Nonpolar metabolites, such as phenolics, sterols, esters, long chain alcohols, and long chain carboxylic acids, were higher in lyophilized feces compared with fecal water. Amino acids and carbohydrates were lower in lyophilized feces compared with fecal water *(105)*. Freeze-drying can provide for greater precision in mixing of dry stool material with solvents and derivatization agents (enhancing reproduc-

ibility). However, concerns have been raised that certain molecular classes, such as short chain fatty acids (acetate, propionate, and butyrate) can be adversely affected by lyophilization. Such concerns may need to be addressed by aliquoting samples, identifying specific molecular targets, and applying specific preservation techniques (e.g., HCl) for specific target molecules (e.g., butyrate) to those aliquots. For instance, attention to the volatile molecular class has been addressed by transfer of aliquots from bulk specimens to head space vials *(106)*. Additional work will be required to determine optimum processing techniques for targeted and untargeted analyses.

One means to address sample stability is centrifugation, in some cases, followed by filtration. The centrifugation (and filtration) step is intended to remove cellular components, which has the effect of substantially reducing active biochemical processes in the sample and their attendant contribution to ongoing molecular flux within the sample. This is a crucial step in deriving fecal water from bulk feces. Although centrifugation and filtration (Table 3) may prevent continuation of metabolic processes because of cellular activity, it may also result in reduction of molecular feature diversity.

Storage of Feces

There is a paucity of data on the long-term storage of fecal samples for metabolomics purposes. However, examination of fecal microbiome samples used to study microbial DNA integrity and fecal protein is informative. Kia et al. freeze-dried human fecal samples, stored them for 14 years at −20 °C, and compared the DNA integrity with freshly collected human fecal specimens *(107)*. The DNA from the retained extracts was more sheared than that of fresh samples, but it was still of sufficient molecular weight to support amplicon-based studies. Morris et al. *(108)* examined protein content and protease activity on fecal samples stored for up to 1 year at −20 °C and −80 °C, and in a variety of storage buffers, and showed that extracted protein was not stable and activity was lost, even with a suitable storage buffer. The most robust solution was to store the proteins in an intact frozen native fecal matrix and extract at the time of assay or analysis. This approach was shown to be appropriate for samples that had been frozen for 1 year and that possessed low levels of protease activity *(108)*. In contrast to the above, Gratton et al. observed notable stability differences between whole feces and fecal water, with fecal water displaying the greatest stability *(97)*.

Freeze–thaw cycles are generally problematic in metabolomics studies *(42)*, and examination of the fecal metabolome is no different. According to Cardona et al. *(109)*, if frozen fecal samples are going to be transported, it should be done as quickly as possible, preferably on ice, to avoid a thawing event. As noted previously, the gold

standard is to freeze samples at −80 °C within 30 min. However, Gratton et al. have argued that if a freeze–thaw cycle might be introduced during the transport, refrigeration for the first 24-h postcollection may be preferred, followed by freezing on arrival at the sample destination (97). Distribution of raw samples into aliquots before freezing or processing is a reliable means to avoid future freeze–thaw cycles, but an initial freeze–thaw cycle during transport should be avoided.

Given that gastrointestinal bleeding is commonly encountered in the clinic, there is a growing interest in the extent to which fecal occult blood may affect the analysis and interpretation of the fecal metabolome. Recently, principal component analysis revealed that occult blood (1 mg Hb/g feces) exerted only a minor effect on the fecal metabolome. However, feces spiked with gross blood (100 mg Hb/g feces) yielded a unique metabotype, suggesting that attention should be given to gastrointestinal bleeding in fecal metabolomics studies (105).

RECOMMENDATIONS

The above evidence supports attention to the following, with a clear need for further development in this field: bulk stool collection, when possible. Recognize that spot sampling may introduce significant metabolite variance. Freeze any sample collected on site within 30 min of sample collection. If transported, do not allow for a freeze–thaw cycle. Some groups advocate that if a thaw cycle is anticipated in transport, samples should be refrigerated rather than frozen on collection. When nucleic acid-based metagenomic analysis and metabolite profiling are a component of the same study, homogenization is also highly recommended. Spot sampling from multiple locations on the bulk stool is recommended, followed by homogenization. Dispense homogenized samples into multiple aliquots to avoid freeze–thaw cycling in future analyses. Avoid >1 freeze–thaw cycle. Short-term storage should be optimally at −80 °C and minimally at −40 °C. Long-term storage should be only at −80 °C. In the analysis, presence of occult blood should be examined. Large-scale studies may want to begin with pilot studies to optimize the method to the research question, while considering the basic principles described herein. Critical among these considerations is whether to use crude fecal samples, fecal water extracts, or lyophilized feces, coupled with consideration for their respective feature yields. If spot testing cards are used, they should be from a homogenate specimen of >1 spot-collection site on the bulk feces specimen. If using lyophilized specimens, collect bulk sample, homogenize, transfer homogenate of sufficient mass (e.g., 100 mg), lyophilize, and transfer to a polypropylene tube for immediate freezing and storage at −80 °C. For fecal water extraction, >15-g sample should be from a homogenate of >1 spot-collection site of bulk feces. Fecal methods are commonly optimized for untargeted metabolomics, but if targeted metabolomics is a component of the study, individual aliquots of homogenate should be prepared in a manner that optimizes recovery of the specific analytes of interest.

Preanalytical Processing and Biobanking of Saliva

Saliva is probably the most commonly collected "other" fluid because it can be collected noninvasively and repeatedly. It has been used in studies ranging from dementia (110, 111) to cancer (62, 112) and oral disease (113, 114). Currently, there are saliva collection protocols in existence, but most biobank protocols are not designed around metabolomics research and alternative metabolomics-specific protocols have not been well validated (Table 2). Given the propensity for both food debris and oral bacteria in saliva samples to both degrade and contaminate the in vivo metabolomics profile (110), protocols should consider this risk at the time of collection and take steps to mitigate it early after the collection period.

There are multiple published protocols for the collection of saliva samples but little published validation of the methods used. Saliva is typically collected either by stimulation with a noxious substance, free spitting, or chewing on a synthetic tampon followed by an extraction procedure (typically centrifugation) to reclaim the fluid. Stimulated collection methods are reported to change the ratio of metabolites to each other (12), and chewing may alter hormone concentrations (115), suggesting unstimulated passive collection as the best approach. Showing pictures to increase saliva production may be beneficial (115).

Before collection, there are various intervals of fasting, tooth brushing, and smoking allowed or documented. Most are in the duration of 1 to 2 h but range from no abstinence recorded (11) to 3 h (116). It is unclear how much effect the length and type of any such restrictions has on the eventual metabolome. Circadian rhythms, smoking, periodontal disease, and fasting states have all been reported as affecting the salivary metabolome (12, 62, 113).

Both no rinsing and rinsing with water before collection are strategies that are frequently used (111, 116–119). Rinsing with water may dislodge and remove the worst food debris in the mouth, but it may also act to stimulate the salivary glands, thus both changing and diluting the sample. Overall, rinsing with water is probably a sensible strategy, although it should be both consistent and recorded in both the standard operating procedures and any subsequent publications.

Handling of the sample both during and after collection will influence the eventual analysis results. The time at 4 °C before freezing ranges from <30 min (120) to <1 days (110). Schipper et al. (121) and Sugimoto et

al. *(120)* have both cited concerns that the salivary metabolome is unstable kept at these temperatures for long periods. It is probably preferable to freeze the saliva after collection in a defined and recorded time window and preferably as soon as possible after collection. Centrifugation before freezing is common *(111, 117, 120)* and is probably recommended because it removes food debris and cellular material before storage *(43)*. However, a systematic evaluation of the optimal time, temperature, and duration for centrifuging is required.

Longer term storage should be at −80 °C or below. Storage at −20 °C has been shown to be suitable for short-term storage for NMR in some limited studies *(12)*, but because degradation of metabolites in other biofluids has been demonstrated to occur at these temperatures *(22)*, −80 °C is preferable if available.

For saliva, not enough evidence exists to recommend one specific protocol over another. Validation of temperature of collection and storage protocols, and especially the effects of storage at 4 °C, should be investigated for metabolomics research. Detailed collection protocols should be drawn up and standardized for any individual biobank and recorded in the methods when publishing new research.

Preanalytical Processing and Biobanking of Cerebrospinal Fluid

The collection and biobanking of cerebrospinal fluid (CSF) has been well reviewed by Teunissen et al. *(122)*, and we support most, but not all, recommendations made in this article. Suggested modifications to their recommendations include centrifugation ($2000g$ for 10 min at 4 °C) before storage to remove and prevent any cells in the CSF lysing on thawing. This step is important even when no obvious erythrocyte contamination is evident *(123, 124)*. Blood and other cells affect the protein content of the CSF, which can affect the metabolite analysis *(125, 126)*. Likewise, following collection, keeping the sample at room temperature for the 2-h period may be too long to prevent the degradation of certain metabolites (e.g., certain amino acids). At a minimum, the time from collection to processing should be both recorded and consistent between samples. Otto et al. *(124)* recommended a maximum of 1 h at room temperature, which appears to be a reasonable compromise. Precentrifuging the samples and removing cellular material may lengthen this time window *(123)*.

Wuolikainen et al. *(22)* looked at storage conditions for CSF. Sublimation of CO_2 will variably affect the pH of samples and, thus, the subsequent analysis. Therefore, we recommend storage at −80 °C or colder in the smallest possible container with a close-fitting screw cap. Regarding collection and storage materials, polypropylene tubes were first proposed for CSF collection because some proteins stick to plastic *(127)*. Polypropylene appears to have now become standard and has been used in a wide variety of metabolomics CSF studies *(122, 128, 129)*. Wuolikainen et al. *(22)* demonstrated both how the material properties of the tubes and the order of tube collection affected the analytical results, but little systematic evaluation of tube material has been attempted for metabolomics. In proteomics, more extensive evaluation has been undertaken, which is broadly reviewed by Willemse and Tuenissen *(130)*, including the propensity for tubes to release their own contaminants into the sample. Overall, when possible, sample tubes should be bought in bulk and the same batch used for an individual study.

Because the first 1 or 2 mL of CSF is the most likely to be contaminated with blood, we support the recommendation that it should be used for medical testing or otherwise discarded, rather than kept for biobanking *(124)*. Any obvious blood-contaminated samples should likewise be discarded.

Because the variation between the ratio of blood concentrations of certain metabolites and the CSF concentrations may be indicative of certain disease states *(131–133)*, consideration as to whether a blood plasma sample should be collected and analyzed alongside the CSF sample should also be determined.

Overall, the existing protocol for CSF collection, when followed, should be sufficient for metabolomics if centrifugation of samples and time to freezing are both considered and optimized. The size and material of storage containers and the temperature of storage are factors that will directly affect analysis results, and there is some evidence that natural diurnal variation may occur in some CSF metabolites, supporting that time of day of collection should be standardized *(134)*.

Preanalytical Processing and Biobanking for Other Biofluids

Other biofluids that have been used for metabolomics include sweat *(135)*, human breast milk *(136)*, lacrimal fluid *(137)*, endometrial, peritoneal, follicular, and synovial fluids *(138–141)*, breath and other volatiles *(142)*, and microdialysates *(143, 144)*. Collection procedures (Table 2) for many of these fluids have not been systematically evaluated, especially in humans. Therefore, we recommend that, until further validation has been conducted, protocols should be adapted from existing protocols for similar metabolomics research.

Some fluid-specific issues are worth highlighting. For breast milk, freezing is a useful way of agglutinating micelles, and thus removing insoluble components from the analysis *(145, 146)*. For peritoneal fluid, endometrial fluid, and menstrual effluent, the World Endometriosis Research Foundation published guidelines based on best-

practice biobanking for these biofluids *(115)*. With current knowledge, the protocols outlined in this review for peritoneal and endometrial fluid are broadly sufficient for most metabolomics research.

For exhaled breath condensate, the American Thoracic Society and the European Respiratory Society published a list of guidelines in 2005 *(147)* on the collection of this type of specimen. This was recently updated as a technical standard for exhaled biomarkers in lung disease *(148)*. We recommend that the metabolomics community follow the suggestions and good practices outlined in this document when collecting samples for any metabolome analysis involving the collection of exhaled breath condensate. Unfortunately, these guidelines do not cover other methods of collecting and storing volatiles.

Ethics of Sample Collection

The issue of biobanking and the wider issue of "big data" introduce some new ethical obstacles, including:

1. New models of consent based around a "known unknown" for both the scientist and the donor about the full implications and uses that the samples may be used for *(149)*.
2. The ability of the data to stay truly anonymous, especially once coupled with other omic or metadata and with an increasing emphasis on sharing data sets *(150)*.
3. Rights and restrictions of the individuals to access their own data, especially when it may affect both their own and their family's health *(151)*.
4. Rights and restrictions of others (e.g., insurance providers, employers, or healthcare companies) to potentially use that data for commercial or alternative purposes *(152)*. The establishment of commercial biobanks is an interesting development that raises new questions in this area.
5. The legality, ability, and ethics of biobanks and researchers sharing biosamples across international borders *(153)*.

Harmonizing collection procedures, ethical consent processes, and documents between biobanks is possible and has been demonstrated to be effective and may enable more effective use of resources *(153)*.

Conclusions

Sample collection procedures must be carefully considered when biobanking for metabolomics studies. Substandard treatment of samples during the preanalytical phase can add substantial variation or bias that may result in misleading analytical results. Procedures that are based on well-validated and evidence-based methodologies should be followed whenever possible, although practicalities sometimes play a role in the final procedure adopted, and the burden on both the volunteer donating the samples and the staff collecting them needs to be considered. Comprehensive staff training is a worthwhile endeavor, so that every member of the collection and storage team appreciates the importance of rigidly adhering to the protocol, especially because consistency in implementing that protocol is an important tool in contributing to reproducibility of results. Once a robust protocol has been developed and defined, it can then be used for future collections. Hence, it is worthwhile taking the time and effort to implement a well-validated protocol from the start. Targeted methods may require protocols specific toward the metabolites of interest. Any reported metabolomics study should define in detail the sample collection conditions as part of the method in accordance with good scientific practice. Peer reviewers and journal editors can support this practice by requesting this information when reviewing submissions.

Review

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