Collection of Urine Samples Using Metabolic Cages or Manually by Spontaneous Urination

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Abstract

The development of minimally and non-invasive sampling techniques to measure current "gold-standard" biomarkers and assess the potential of a new generation of biomarkers is central to future-proofing animal research. Urine can be sampled non-invasively and is easy to voluntarily collect from pigs in confined (e.g. metabolic cages) or open (group or single pens, outdoors) environments. Urine has been utilised in a wide range of animal studies to measure a plethora of parameters, including untargeted and targeted metabolites, clinical chemistry parameters and hormones. Here we present protocols for the collection of urine from pigs housed in metabolic cages, as well as from unrestrained pigs. The metabolic cage method is ideal for continuous sampling, whereas the manual collection by spontaneous urination is ideal for spot sampling in open environments. This SOP provides recommendations for the following: 1) materials for sample collection, 2) step-by-step procedures for collecting samples from pigs and 3) initial urine sample processing steps. The sampling techniques described in this SOP provide reliable methods for the collection and initial processing of samples for the measurement of a range of analytes in pig urine, thereby promoting the development of minimally invasive approaches to measuring biomarkers in animal research.

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Keywords

Pigs · Sample collection · Sample processing

5.1 Introduction

Urine is an important biological sample that has been used in biomedical, veterinary and nutritional research, to investigate a wide variety of research questions (Czech et al. 2022; Kim et al. 2020; Nixon et al. 2020; Tkaczyk et al. 2021). In pigs, urine has been used to:

(1) Assess kidney function. It can be used to measure the renal clearance of certain substances, such as creatinine, urea and insulin (Dhondt et al. 2020; Jia et al. 2010).
(2) Monitor metabolism to study the effects of a treatment or a diet (e.g. protein-rich diets, medications or probiotics), the nitrogen balance (ATOL_0005338) or detect metabolic imbalances (Berghaus et al. 2023).
(3) Study pharmacokinetics to quantify the excretion of substances and their metabolites, assess their bioavailability and study the half-life of a compound (Dhondt et al. 2020).
(4) Measure specific biomarkers and monitor animal health (ATOL_0000928) to diagnose urinary tract infections, track the progression of certain metabolic or inflammatory diseases and detect the presence of abnormal or pathological molecules (Svoboda et al. 2024; Sachse et al. 2016).

There are several methods for collecting urine, including cystocentesis, bladder catheterisation, metabolic cages (EOL_0002023) or manual urine collection by spontaneous urination (ATOL_0000802). The choice of method depends on the type of analysis to be performed, with the caveat that all methods differ in their degree of invasiveness. Cystocentesis and bladder catheterisation are the ideal methods for collecting sterile samples for bacteriological analysis. However, these are highly invasive procedures that carry a risk of infection and require technical expertise. Urine can also be collected using non- or minimally invasive methods, making it a potential candidate to replace more invasive sampling techniques, if and when appropriate urinary biomarkers are found. The two methods described here, metabolic cage and manual urine collection by spontaneous urination, both allow for non-invasive urine sampling.

Metabolic cages are the "gold standard" method when sequential urine samples are required or when a large volume of urine needs to be collected over a particular period. For example, during metabolic and nitrogen balance studies, it is essential to know how much urine is produced over several consecutive days. Unlike faeces, which can be collected as a spot sample by incorporating indigestible markers into the feed (Chap. 6), there are currently no markers in urine to measure total output. Metabolic cages also require significant financial investment, as experiments involving these cages necessitate rooms that are large enough to accommodate the equipment and provide sufficient workspace for staff. Additionally, these rooms must be

equipped with systems to control temperature, humidity and water supply. Facilities must also allow for daily cleaning of both rooms and cages, as well as include appropriate waste disposal systems. In addition, their use in the EU is heavily restricted due to the confined nature of their setup, which affects pigs' natural behaviours and can place unnecessary stress (ATOL_0002301) on them. There is currently no age limit on the use of metabolic cages in pigs; studies have been conducted in newborn piglets and in sows over 4 years old. In contrast to metabolic cages, manual urine collection by spontaneous urination can be conducted while pigs remain unrestrained in their normal housing environment. However, the method requires staff to remain in the housing environment and wait for pigs to urinate. This makes manual urine collection by spontaneous urination a time- and personal-intensive method.

To ensure the reliability of urine-based measurements, minimizing sample contamination is critical. This includes avoiding faecal contamination and environmental debris. Proper handling and collection protocols, such as using clean sampling equipment and promptly transferring samples to appropriate storage conditions, are also important. Additionally, reducing stress, which can influence urinary biomarkers, by habituating animals to the sampling environment (metabolic cages) or to the presence and proximity of human collectors (manual urine collection by spontaneous urination), helps to ensure that the data reflect true physiological conditions rather than stress-induced changes.

5.2 Goal and Scope of the Procedure

The goal of this SOP is to provide detailed information on the collection, processing and storage of urine samples collected from pigs under the following conditions: (1) individual housing from the post-weaning period onwards in metabolic cages (Sect. 5.3) and (2) individual or group-housing, in pigs of any age, using the manual urine collection by spontaneous urination method (Sect. 5.4).

These guidelines on urine collection have been designed to (1) reduce sample contamination (i.e. from faeces or feed), (2) be easy and convenient to conduct, (3) enable reproducibility, (4) habituate pigs to urine collection, and (5) minimise the stress, pain (ATOL_0000863) or discomfort experienced by the animal, particularly when using metabolic cages.

5.3 Urine Collection from Pigs Housed in Metabolic Cages

5.3.1 Materials and Equipment

5.3.1.1 Metabolic Cage

- Should be adjustable in width and length according to the size of the pig (Fig. 5.1a-c).
- Should be height-adjustable for ease of use by the people working with the pigs.

Fig. 5.1 Adjustable metabolic cages used at (**a**) FBN (Credit: Mariagrazia Cavalleri, FBN) and (**b**) Agroscope, with an example of (**c**) a feeding and water system (Credit: Marion Girard, Agroscope)

- Should have a catchment tray or a gradual slope design for catching the urine samples.
- Filter(s) need to be installed at the urine collection point of the floor and/or tray to prevent faecal and feed matter from entering the urine collection vessel (Fig. 5.2a-c).

5.3.1.2 Urine Collection Containers

- The size of the collection container (~1–4 litres) to be used will depend on:
- The size of the pig (bigger pigs produce more urine). A weaner pig (10–15 kg) produces 0.5–1.5 litres per day, whilst a growing-finishing pig (50–100 kg) will produce between 1.5 and 8 litres per day.
- The sampling time duration. In pigs between 20 and 40 kg, a 1 litre bucket is sufficient to collect urine over 3- to 12-h time periods with no preservatives or pH regulators, for the measurement of Carbon-13 (^{13}C) (Cavalleri et al. 2025), mannitol and lactulose. In certain metabolic trials, it is important to know the

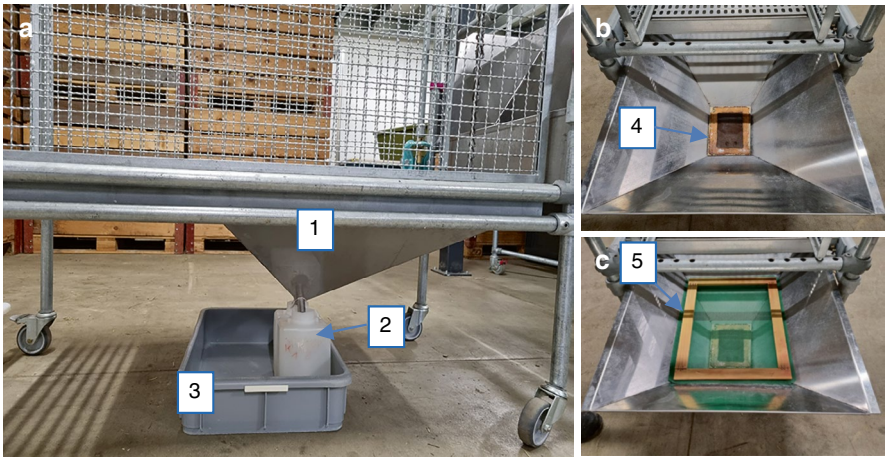

Fig. 5.2 Example of a urine collection system (**a**) [1] Stainless-steel collection tray, with [2] Urine collection container (~2 L) and [3] Container that can be filled with ice to keep the urine sample cold, and top-down views of the stainless-steel collection tray (**b**, **c**) with [4] Secondary 0.5 mm filter, and [5] Primary 2 mm filter (Credit: Marion Girard, Agroscope)

volume of urine produced over 24 h in order to determine the amount of a compound excreted in the urine. For the analysis of nitrogen excreted over 24 h in nitrogen balance studies, urine is collected into two 1.5-litre containers (one acidified and one non-acidified) using a collection tray with two outlets. The fill level of these containers is checked once or twice during the day. If containers are found to be 2/3 full, they must be replaced with new, clean and dry containers.

The intake of the collection container (where the urine enters) should be designed to reduce exposure to the external environment and thus reduce any potential environmental contamination.

- We recommend using 1 litre plastic buckets covered with a stainless-steel round sieve (Rotilabo, Product ID: 8099.1, Roth Labs) that has a 1.5 mm mesh to catch any large environmental particles and acts as a secondary filter for the urine samples. This design has been used for the measurement of ^{13}C enrichment (Cavalleri et al. 2025) and concentrations of lactulose and mannitol (markers of intestinal permeability).
- An alternative option is to use a container with a narrow opening at the top. You should ensure that the end of the collection tray fits properly into the opening of the container, as shown in Fig. 5.2a.

At the end of each experimental period, containers should be cleaned in a dishwasher to ensure thorough cleaning. If the containers do not need to be sterilised, rinsing with hot soapy water and drying may be sufficient. The frequency with which the urine collection containers should be cleaned depends on the trial design and what needs to be measured.

- An example is a study conducted over a 36-h experimental period to measure the ^{13}C abundance in urine, using ^{13}C stable isotope labelled lactose-ureide (Cavalleri et al. 2025). Samples were collected over a 12-h period at 3-hr intervals (0–3, 3–6, 6–9 and 9–12 hr) prior to lactose-^{13}C-ureide administration and from 12 to 24 h post-lactose-^{13}C-ureide administration. After each sampling time-point, the urine collection buckets and stainless-steel round sieves were washed with hot soapy water to remove any residual ^{13}C, immediately before the next urine collection period. As ^{13}C was the only parameter being measured, the urine collection buckets did not need to be sterile and could be reused.
- As another example, if the urine of one pig needs to be collected over several consecutive days, it is advisable to wash the containers with hot water and dry them between each day of collection and to use the same containers for the same animal each day. In the case of nitrogen balance studies, it is advisable to have two containers (one acidified and one non-acidified) to place under the collection tray and two spare containers (one acidified and one non-acidified) if urine production is abundant.

5.3.1.3 Other Materials and Equipment for Urine Sample Collection in Metabolic Cages

- Water-resistant felt-tip pen.
- Lab gloves (latex or nitrile).
- Scale, to weigh the urine collected.
- Styrofoam container with ice to place the bucket/container in; this keeps the collected urine cold and slows down bacterial degradation of target metabolites (Fig. 5.2a).
  - The size of the Styrofoam container and amount of ice to be used will depend on many factors. Some examples are as follows: the length of time urine is collected (longer duration requires a larger container and more ice) and environment (temperature-controlled vs. non-controlled stalls). We suggest the use of thermometers placed in the ice next to the collection container to ensure the temperature is maintained below 4 °C.
  - Ensure that the container can always be easily removed from under the collection tray, even when it is full and submerged in ice. This is especially important when a new container needs to be used.
- Urine preservatives may need to be added for analysis of specific metabolites.
  - For example, to measure nitrogen, it is recommended to acidify urine with sulfuric acid to prevent microbial growth. We recommend adding 25 g of sulfuric acid at 3 M to 1.5-liter collection containers.

5.3.1.4 Other Materials and Equipment for Urine Sub-Sample Preparation in Metabolic Cages

Urine is typically collected in buckets/containers (~1–4 litres), and the large volume produced requires sub-sampling into smaller aliquots, using the following equipment:

- Styrofoam container with ice to transport the sub-samples.
- Lab gloves (latex or nitrile).
- Pipettes and tips (volumes will depend on how much urine is being sub-sampled), to prepare the sub-samples.
- Eppendorf tubes or 1-litre containers for the sub-samples (if needed).
- Some studies require a small aliquot, while others require an aliquot representative of the entire collection period. One example would be collecting 2% of the total daily urine excretion over several days. In this case, larger containers (0.5–1 litre, one per pig) are needed.
- Centrifuge, with 4 °C cooling.
- Freezer (−20 °C or − 80 °C), to store the sub-samples until further analysis (if needed).

5.3.2 Prerequisites and Preparation

5.3.2.1 Metabolic Cage Setup and Study Design

Metabolic cages should be in a room where multiple cages can be set up, so that the pigs can hear, see and smell each other. One design is a six-cage setup, with two rows of three cages set across from each other (Cavalleri et al. 2025). The room should also have adequate ventilation and climate control so that the pigs are kept under temperature and humidity conditions according to their age and development stage. For pigs between 20 and 40 kg, we recommend a room temperature of 22 °C and a humidity of 40–60%. In addition, the room should have lighting to control day/night settings. Once the appropriate metabolic cages have been selected, they should be cleaned with either a hot or cold high-pressure water cleaner at least 24 h before use and immediately after the pig has been removed. Optionally, cleaning agents can be used, but local environmental regulations need to be checked to determine which agents are permitted. Prior to pigs entering the metabolic cages, the water supply should be checked to confirm that it is working, so that pigs have free access to water whilst in the metabolic cage (Fig. 5.1a and c). If water metres have been installed, they should be checked for proper functioning, so that water intake by the pigs can be recorded.

How long an animal stays in the metabolic cage will depend on your experimental design and aims. As some general guidelines, under EU Regulation: Directive 2010/63/EU (for scientific use), there is no maximum time that pigs can be held in metabolic cages. Instead, the system is classified by severity, as follows:

- Short-term (< 24 h) is classified as severity degree 1 (non-harmful/mild).
- Moderate durations (up to 5 days) are classified as severity degree 2 (moderate).
- Long-term (> 5 days) are classified as severity degree 3 (severe).

The Swiss Animal Protection Ordinance also includes a minimum recovery period for stays in metabolic cages, depending on how long the animals are kept in a metabolic cage and the level of stress caused by the experiment (Federal Food

Safety and Veterinary Office, 2017). For a stay in metabolic cages of up to 8 h, the minimum recovery period is at least 16 h. For stays from 8 to 24 h, the minimum recovery period is 6 days. For stays from 4 to 7 days, the minimum recovery period must be the length of the detention period plus 14 days.

There are also no EU regulations regarding space allowance in a metabolic cage. Rather, space allowance is determined by the size of the pigs and the study design. Some studies require the movement of pigs to be restricted to standing and sitting. This requires that the sides, front and back of the metabolic cage are adjusted inwards so that the pig cannot move forward, backwards or turn around. This is important when urine needs to be collected while minimizing the potential for any faecal contamination, for example, in nitrogen balance studies, where the nitrogen excreted in the urine is quantified. Another example would be studies that use metabolic cage-mounted infusion pumps. During these studies, pigs receive continuous intravenous infusions, and it is critical that the animals are unable to pull the catheter out (Rasch et al. 2021). However, such a restriction is only recommended for the infusion period. Once the infusion period is complete, the sides of the metabolic cage should be adjusted, allowing the pig more movement. In other studies, pigs do not need to be restrained in the metabolic cage. An example would be studies where data on faecal and urine output needs to be collected whilst blood (ATOL_0005631) samples are collected from indwelling catheters that exit on the back of the pig and are fixed to the pig by bandaging to prevent them from being pulled out (Cavalleri et al. 2025). In general, when contamination of urine with faeces is a major concern, the use of males (castrated or uncastrated) is recommended over females, since it is easier to minimise the contamination of urine with faeces in males.

Important

In the end, the type and size of the metabolic cage, as well as the identity of subjects and severity grade and duration of restriction, will be determined by the researchers based on the goals of their study. However, we recommend that researchers design their studies to minimise the time that pigs spend in metabolic cages while ensuring robust data collection. Any metabolic cage study beyond 5 days constitutes severe restriction by EU law and requires rigorous ethical justification and oversight.

5.3.2.2 Animal Adaptation to the Metabolic Cages

Habituation to the metabolic cage is vitally important to the health and welfare (ATOL_0000765) of the animals used in any study, as animals that are well adapted have reduced levels of stress, and can be easily handled during the experimental period. Here we provide some examples of habituation techniques, based on studies conducted by our research groups. However, we would like to emphasise that these are only examples. The adaptation protocols used for any metabolic cage study will vary depending on the study's specific requirements. For example, in a study with catheterised pigs, weighing between 20 and 40 kg, the pigs were first housed in

individual stalls with transparent walls, so that the pigs could see each other. The individual stalls contained enrichment devices (EOL_0001921, balls, chew toys, ropes, etc.), and twice a day, staff entered the stalls to check catheters and play with the pigs. The pigs were then habituated to the metabolic cages three times over a 1-week period. On habituation days, the subjects were moved to metabolic cages at 7:00 and kept in the cages until 16:00. Pigs were then fed their morning meals at 7:30 in the cages.

As another example, in a balance study, urine was collected from the same animal at two different stages over 5 consecutive days: once during the growing period (around 40 kg) and once during the finishing period (around 80 kg). Before placing the animals in metabolic cages, they were kept in groups for at least two weeks. During this time, the animals habituated to their companions and to the feed. Any pigs that were reluctant to interact with the experimenter were not selected for the cages. On the day that they were placed in the metabolic cages, several pigs from the same enclosure entered their cages in the morning. Once in the cage, a small food reward was given, followed by the morning feed ration. The first day in the cages was used for additional habituation, and no samples were taken. During their stay in the cages, the pigs received one meal in the morning and one meal at the end of the day. An experimenter checked on the pigs two to three times during the day to ensure that they were eating and drinking properly.

5.3.2.3 Preparation of Urine Collection Containers

- The collection containers should be clean (not necessarily sterile) and leak-proof and preferably have a lid or mesh cover to avoid contamination.
- The materials and surfaces in contact with the urine should neither react with nor adsorb any analytes of interest (Delanghe and Speeckaert 2014; Kiyokawa et al. 2011). In addition, they should be free of interfering substances and particles. For instance, albumin binds to the surface of some plastics, which may lead to poor recovery at low concentrations (Hara and Shiba 2003). Plastic (e.g. polyethylene or polypropylene) or glass containers may be used for urea or nitrogen analyses. Additional requirements, such as amber-coloured containers to analyse light-sensitive analytes (such as porphyrins and urobilinogen, for instance), may be needed.
- If required, carefully label the collection containers with an indelible marker. The labels should resist water, urine and/or acid.
- In some studies, such as balance studies, it is important to know the exact volume and/or weight of urine collected and to use a preservative solution. For this, it is advisable to prepare the collection containers 1 or 2 days before sampling begins.
  - Prepare a spreadsheet containing the weight of all empty containers. Decide whether or not to include the weight of the cap in this calculation.

  Then, prepare the preservative solution (if required). For example, 3 M sulphuric acid can be used to acidify urine in order to determine its nitrogen content. Add 25 g of this solution to the containers and note the exact weight on the sheet (accuracy: 0.001 g). As concentrated sulfuric acid is highly corrosive, it

should always be diluted by adding the acid to water rather than the other way around. Wear gloves, safety goggles and a lab coat, and use acid-resistant containers.

5.3.2.4 Preparation of Urine Sub-Sample Collection Tubes/ Containers

- Carefully label the sub-sample collection tubes/containers with an indelible marker. The labels should resist water, urine and/or acid.
- If the study requires a urine sub-sample that is representative of the entire collection period (e.g. 2% *w/w* of the total daily urine excreted), create a spreadsheet to calculate the necessary urine weight to be aliquoted.

5.3.3 Description of the Procedure

5.3.3.1 Daily Sample Collection

1. Mount the urine collection tray under the metabolic cage.
2. Set up the urine collection system, by setting up the filter system and then the collection container(s) below the collection tray (Fig. 5.2a-c).
3. According to the metabolites that need to be analysed, it may be necessary to:
4. Place a refrigerated system below the collection container (e.g. with an icebox that might be changed frequently according to the room temperature—Sect. 5.3.2).
5. Place a container containing a preservative solution (Sect. 5.3.2).
6. If urine is being collected throughout the day, check the volume collected once or twice a day. If the container is two-thirds full, wear lab gloves and replace it with a spare one that is clean and dry, to prevent dilution of the urine content.
7. Record the volume and/or the weight of urine collected on a spreadsheet if this is required.

5.3.3.2 Collection over Several Days

In particular, for balance studies, urine must be collected over several consecutive days (e.g. 5 days). For the first collection day, follow Sect. 5.3.3.1, steps 1–5. On the second day:

1. Remove the containers from the collection system.
2. Clean the metabolic cages with water; this helps to limit contamination with faeces. Pigs remain in the cages during the cleaning.
3. Replace the full urine collection containers with new, clean, dry containers.
4. Weigh all the containers (non-acidified and acidified, if applicable) to determine the amount of urine produced in 24 h. Determine the amount to be aliquoted (e.g. 2% w/w of the total urine produced) and record it on the spreadsheet.

5. Gently shake the container to ensure that the urine is mixed evenly. Weigh the aliquots in 0.5–1 litre containers (Sects. 5.3.1.4 and 5.3.2.4), and freeze them immediately at −20 °C.
6. Once the aliquots have been collected, wash the urine collection containers with hot water and dry them with paper towels or a cloth so that they can be used the next day.
7. On the subsequent collection days, follow the same steps as on the second day of collection. After weighing the urine, place the aliquots in the 0.5–1 litre containers (one per pig) that were frozen the previous day and freeze them, thus creating a pooled sample for each pig.
8. Check the volume of urine produced once or twice a day. If the containers are two-thirds full, remove them, close them with a cap and leave them in the larger container (see the grey container (3) in Fig. 5.2a). Then, replace them with clean, new containers.

5.3.3.3 Sub-sampling and Storage

1. Collected urine samples should be mixed by holding the container and moving it around to ensure that they are homogeneous.
2. The urine should then be pipetted into new clean tubes (Sects. 5.3.1.4 and 5.3.2.4) and centrifuged according to the experimental protocol required. For the measurement of ^{13}C enrichment, lactulose and mannitol, urine samples have been centrifuged at 1000 × g for 10 min at 4 °C. For the measurement of cortisol (ATOL_0005350), creatinine, amino acids, amino-metabolites, nitrogenous waste products and markers of protein catabolism, urine samples have been centrifuged at 3000 × g for 10 min at room temperature. Centrifuging helps to remove particulate matter that could interfere with sample measurement.
3. Supernatants should then be sub-sampled to tubes with an appropriate volume for planned downstream analyses (Sect. 5.3.1.4). Downstream analyses may include the measurement of: (1) ^{13}C enrichment using isotope-ratio mass spectrometry (Cavalleri et al. 2025); (2) lactulose, mannitol, amino acids and/or amino-metabolites using HPLC; (3) cortisol or other hormones using enzyme-linked immunosorbent assays; or (4) creatinine, markers of protein catabolism and/or nitrogenous waste products using colorimetric and enzymatic assays.
4. If tubes are to be frozen, fill them up to two-thirds of the total tube volume to prevent them from bursting due to volume expansion during freezing. We recommend storing at −20 °C for up to 3 months and at −80 °C when storing for more than 3 months.
5. To obtain a representative sample of the total collection period when collecting urine over several consecutive days, weigh the amount of urine produced per day (Sect. 5.3.3.2) and collect a fixed percentage (e.g. 2% *w/w*). Since the amount of urine produced varies from day to day, this allows for standardisation. Record these amounts (total amount produced and amount collected) on a spreadsheet, then freeze the sample at −20 °C. Then, fill this same tube with the subsequent days' aliquots, i.e. use a single tube for all days as described in Sect. 5.3.3.2. Just

before analysis, thaw the aliquots, collect a portion in a new tube (the volume depends on the analysis) and centrifuge it as described above.

5.4 Manual Urine Collection by Spontaneous Urination from Unrestrained Pigs

5.4.1 Materials and Equipment

5.4.1.1 Housing

Pigs can be housed either individually or in groups. The size of the pens depends on each country's legislation. Examples of housing for pigs in different categories, both individually and in groups, are shown in Fig. 5.3. Pigs must have ad libitum access to fresh drinking water for the entire duration of the experiment. They prefer drinking from a trough (EOL_0001618), rather than a nipple drinker. Be sure to change the water regularly especially in the summer. Also, free range sows must have access to a mud hole, when temperatures reach above 15 °C. Otherwise, they might bathe in the water trough and contaminate the drinking water.

Fig. 5.3 Examples of pig housing systems where the manual urine collection by spontaneous urination method can be used: (**a**) free-range system for lactating sows (Credit: Maria Eskildsen, Aarhus); (**b**) individual system for lactating sows (Credit: Marion Girard, Agroscope); (**c**) group system for fattening pigs (Credit: Marion Girard, Agroscope)

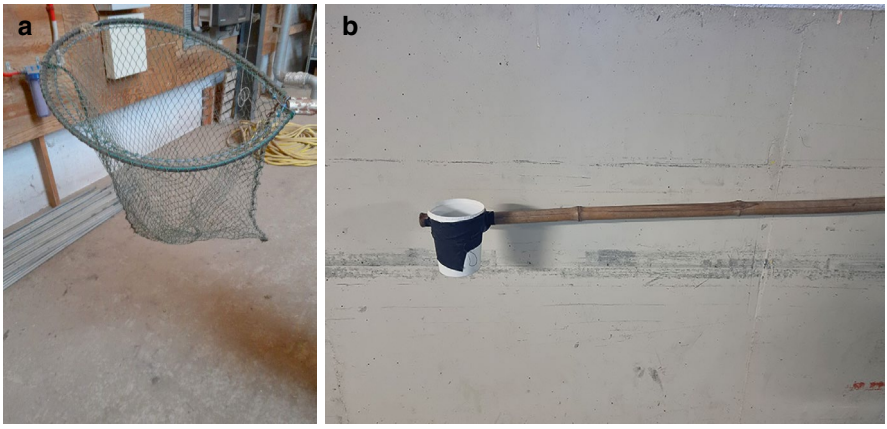

Fig. 5.4 Examples of home-made urine collection systems: with a fishing net (**a**) in which a collection bucket can be placed, or a stick with the collection cup attached with adhesive tape (**b**) (Credit: Maria Eskildsen, Aarhus and Marion Girard, Agroscope)

5.4.1.2 Materials and Equipment for Urine Sample Collection

- Urine should be collected in a bucket or cup with a minimum capacity of 500 mL. If the animal is some distance away, homemade devices such as a net to hold the bucket, or a stick with a collection cup attached (Fig. 5.4), can make collection easier.
- Clean collection containers (preferably sterile).
- Long-handled ladle or scoop.
- Disposable gloves.
- Protective clothing and boots.
- Labels and waterproof markers.
- Cooler with ice packs for sample storage.
- Disinfectant for cleaning equipment.

5.4.1.3 Materials and Equipment for Weighing and Urine Sub-Sample Preparation

- Refer to Sect. 5.3.1.4 for details of the materials required for sample processing, including weighing (if needed) and sub-sampling.
- Eppendorf tubes or 50–100 mL tubes/containers for the sub-samples.
- Urine preservatives may need to be added for analysis of specific metabolites.
- For example, to stabilise pH and prevent volatilisation when nitrogen is analysed, we recommend adding 0.5 mL of sulfuric acid at 1 M to 50 mL urine sub-sample tubes. This results in a pH < 2, which stabilises some urinary metabolites, such as nitrogen, when this is necessary for specific analyses.
- Pipettes and tips (volumes will depend on how much is being sub-sampled), to prepare the sub-samples.

5.4.2 Prerequisites and Preparation

5.4.2.1 Description of Different Housing Conditions

Pigs may be housed in free-range housing systems under certified organic conditions in accordance with EU regulations (EU 2018/848). During gestation, several sows may be housed together in common huts. During lactation, each sow should be housed individually with her litter in outdoor farrowing huts, which provide shelter, thermal protection and the opportunity for undisturbed maternal behaviour. These huts are distributed across spacious paddocks with continuous access to pasture, enabling the expression of natural behaviours such as foraging, rooting and exploration. Each free-range unit consists of one sow and her piglets, to prevent inter-sow aggression and to facilitate natural nursing and bonding. Outdoor paddocks are maintained with sufficient vegetation cover to support voluntary intake of grass, and the layout of the facilities allows for reliable assessment of feed intake (ATOL_0000772), energy expenditure through locomotive activity and thermoregulatory demands (ATOL_0000857). The water trough should be full and fresh drinking water should be available at all times.

When working with lactating free-range sows, EU law (EU 2018/848) requires that huts are arranged in such a way that the animals can see each other. Huts should be closed in advance before dawn, as the sows will urinate first thing in the morning. Close the huts quietly, as too much noise will wake them up and possibly cause them to urinate within the hut. When collecting urine from pregnant sows in common huts, it is important to have enough stock personnel (one person per sow) or to adjust the number of sows that are let out of the hut at the same time to the number of people available. The sows will likely urinate at the same time, and one person will not be able to collect from more than one sow. It is very time-consuming to collect urine from outdoor pigs at times of the day other than in the morning. Urine can also be collected from pigs in more conventional housing conditions. As with free-range animals, it is advisable to be present in the morning to collect the samples, since animals often urinate shortly after waking up.

5.4.2.2 Animals

Urine can be collected from males and females of any age. If it is not possible to collect in the morning, it is advisable to monitor the pig's behaviour and anticipate urination, which will often happen after feeding or drinking. The procedure is non-invasive and free of pain for the animals. However, some pigs might be stressed by being followed by a person for 5–10 min. To reduce the stress level of the animals and minimise the risk of attack (especially in lactating sows), it is recommended that pigs become habituated to the experimenters. To achieve this, 1 to 2 weeks before collection begins, experimenters should spend at least 10–15 min a day in the pens with the animals, observing them. When the animals allow them to approach, the experimenters should try to stroke, pat and interact positively with them. Observing the animals for longer periods can also help to identify where and when they urinate. The experimenter may also enter the pen with the container that will be

used for collection and attempt to take samples, which will then be discarded. If an animal shies away from human contact, the collection container can be mounted on a 2-metre-long stick with adhesive tape or be placed inside a fishing net (Fig. 5.4). If this system is to be used, it must be introduced into the pens during the habituation period and tested before the collection phase begins. If pigs are reluctant to urinate, it can help to lead them gently to the defaecation (ATOL_0000492) area where they usually urinate.

5.4.2.3 Preparation of Urine Collection Containers and Sub-Sample Tubes

The bucket or collection container (cup with or without fishnet/stick method) should be clean (not necessarily sterile) and leak-proof.

The bucket or collection container and the sub-sample tubes should be labelled with an indelible marker, which can resist being rubbed off by movement (such as that experienced when carrying the bucket behind the sow before she urinates), water, urine and/or acid.

If the volume or weight of the collected urine needs to be measured, the weight of each container must be recorded.

If the metabolite you analyse requires a chemical preservative after collection (e.g. for nitrogen analysis), put it in the sub-sample tubes. For example, by adding 0.5 mL of sulfuric acid at 1 M to 50 mL urine sub-sample tubes as mentioned in Sect. 5.4.1.3. As concentrated sulfuric acid is highly corrosive, it should always be diluted by adding the acid to water rather than the other way around. Wear gloves, safety goggles and a lab coat, and use acid-resistant containers.

5.4.3 Description of the Procedure

5.4.3.1 Sample Collection

The concentration of nutrients and urine volume can vary considerably depending on weather, dry matter in the feed (grass/roughage), amount of water drunk, etc. As previously mentioned, it is also recommended to collect the sample when the pig wakes up, as it is more likely that pigs will urinate soon after waking up.

5.4.3.2 Procedure

Important
Do not collect urine from pigs alone, as there are risks of injury if animals become aggressive (ATOL_0000914). However, thorough habituation can help to minimise this risk.

1. Take the bucket or the collection container. If needed, set up the urine collection system (e.g. mount the collection stick and then the collection container below).

2. Have enough staff available in case the animals all urinate at the same time. It is also a good idea to have one person responsible for processing samples while the others collect urine.
   a) In a free-range system, one person should open the hut while others are assigned to each sow. After opening the hut, each person should follow a pig at a 2–3-metre distance. The pigs will most often urinate within the first five minutes after leaving the hut in the morning (Fig. 5.5a).
   b) Indoors, pigs kept in groups often urinate in the same area of the pen. In this case, it is sufficient to follow the pigs at a distance of 1–2 metres, especially when they approach this area.

Fig. 5.5 Manual urine collection by spontaneous urination method in free-range sows (**a**) and fishing net used to hold bucket with container for urine collection in group housing (**b**, **c**) (Credit: Maria Eskildsen, Aarhus)

3. When a sample has been collected and the collection bottle needs to be changed between animals, wear lab gloves (nitrile or latex) and remove the collection bottle. Replace with a new collection bottle that is clean and dry to prevent dilution of the contents.

5.4.3.3 Sub-sampling and Storage

- Mix the urine in the collection bottle by shaking the bottle, and then use a pipette to transfer around 1 or 2% of the total amount of collected urine into labelled tubes of 2–100 mL.
- We recommend centrifuging samples at 3000 × g for 10 min to remove particulate matter. Then, aliquot the supernatant into sterile tubes.
- Several tubes can be used if several analyses are required.
- If tubes are to be frozen, fill them only up to two-thirds of the total tube volume, to prevent them from bursting due to volume expansion during freezing. We recommend storage at −20 °C for up to 3 months and −80 °C for more than 3 months.

5.5 Compliance with the 3R Principles

The metabolic cage protocol outlined in this SOP requires that the pigs be restricted for an extended period with minimal movement, which is likely to negatively impact on their welfare. However, metabolic cages are currently one of the only techniques that allow for repeated and continuous non-invasive urine collection from one animal, whereas this is not currently practical in the spontaneous urination system. Metabolic cages thus reduce the number of animals required for studies, where the exact volume of urine produced per animal is required, since alternatives under open housing systems require using a larger number of animals to obtain accurate estimates of daily urine volumes. In addition, multiple tests can be performed in the metabolic cages using the same animal, further reducing the number of animals required and recycling those currently in the study.

The manual urine collection by the spontaneous urination method is currently the only technique that allows completely non-invasive urine collection of pigs in a more natural environment. This method is useful when one or two daily samples are sufficient to capture changes in analytes of interest. However, the method is less useful for continuous collection of urine, i.e. throughout the day, as this requires enough staff to be on site at all times, which is often impractical. In addition, the constant staff presence may disrupt the natural behaviour of the animals. Your experimental protocol needs to clearly justify the nature of the sample collection required and thus the method to be used (i.e. metabolic crate vs. manual urine collection by spontaneous urination).

Table 5.1 Advantages and disadvantages of using metabolic cages or open environments for urine sampling

Method	Advantages	Disadvantages
Metabolic cages	Easy separation of urine and faeces Can perform repeated and continuous sampling of urine Non-invasive Enables total daily collection	Acclimation needed Risk of faeces contamination when using female animals Welfare concerns due to lack of space and social isolation
Manual urine collection by spontaneous urination	Easy separation of urine and faeces Non-invasive No restraint needed Allows sampling in home environments and in social groups	Time- and personnel-intensive Difficult to continuously sample animals Greater risk of physical injury to personnel

5.6 Conclusions

This SOP outlines two minimally or non-invasive procedures for collecting urine. While this SOP is primarily based on examples from metabolic studies, it is also applicable to other fields where the analysis of analytes in urine is relevant. Metabolic cages are currently one of the only techniques that allow continuous, sequential sample collection, whereas manual urine collection by spontaneous urination allows for sporadic collection under more natural conditions. The advantages and disadvantages of the procedures outlined in this SOP are summarised in Table 5.1.

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