



# Guide to preparation of liquid biopsies for nucleic acid extraction

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# 1 Guidelines on experiment design

A successful experiment starts with a good experiment design. Based on our extensive experience in analysis of nucleic acids, we recommend to take the following considerations into account in the design of your experiment:

## 1. Minimize confounding variables and noise

Eliminate variances in materials, procedures, equipment, and personnel. Examples include using a single lot of reagents and materials, standardization of all labware, and ensuring batch, operator or time point are characterized and variability minimized. In addition, samples should be collected under similar conditions and where possible at the same time, relative to e.g. treatment or food intake. If elimination of these sources of variation is not feasible, ensure these "noise" factors are built into your experimental design.

A Biogazelle project manager will assist you in identifying possible sources of variation and can support you in optimizing your experiment design to cope with the inherent noise factors.

## 2. Collect a sufficient number of samples to statistically support your conclusion

The number of samples required depends on various factors, such as the research question, the data analysis strategy, the magnitude of the measurement difference, and the measurement variability within each experimental group. The latter is inherent to the underlying biology, collection, isolation, and analysis. A pilot study may be required to quantify this variability and provide an educated estimate of the number of samples required.

We recommend an absolute minimum of three biological replicates per group for hypothesis testing: for cultured cells, at least 3 independent replicates should be included; for inbred organisms (e.g. lab mouse) at least 6 animals per group are advised; for human individuals at least 12 individuals per group are recommended. In general, to generate robust results, the rule "the more samples per group, the better" applies. As such, for a biomarker study, a typical starting number when working with human individuals, is 30 samples per group.

## 3. Validate each sample prelevation method upfront

Conduct a study on representative samples to ensure your prelevation method maintains the representation, quality, and yield of the RNA or DNA required to draw valid conclusions. This often requires a careful pilot study prior to the main study. Remember, garbage in equals garbage out.

## 4. Provide as many details as possible on the sample prelevation and isolation method upon sample submission.

If a customer provides this information, it will be reported back along with your experimental results so you may track isolation method performance against experimental results. It can also assist the Biogazelle project manager in troubleshooting anomalous results if they occur.

## 2 Collecting and preserving liquid biopsies for nucleic acid extraction

At Biogazelle, liquid biopsies from different sources have been successfully processed. Below, we provide recommendations for the most common body fluids. For specific instructions on less common liquid biopsies, take a look at the general recommendations at the top of the list, or contact the experts at Biogazelle.

### General instructions

- The volume of liquid biopsy required for nucleic acid extraction depends on the sample type and downstream application. We typically start RNA extraction from 0.2 ml and DNA extraction from 1 ml body fluid. Please, contact us if you want to use larger or smaller volumes.
- In order to isolate circulating cell-free nucleic acids from liquid biopsies, appropriate and standardized procedures to separate fluid from cells, particles or debris should be followed prior to freezing. This contributes to high nucleic acid yield and minimizes variation caused in pre-analytical steps. Specific guidelines for plasma, serum and urine are given below. For other fluids, centrifugation for 5 minutes at 3000 g prior to freezing is recommended.
- Store cell-free liquid biopsies at -80 °C in nuclease-free tubes.
- The choice of collection tubes, the collection procedure, the centrifugation (speed, duration and number of steps) and the biology of the sample have an influence on the nucleic acid yield and the analysis and interpretation of downstream results. Therefore, we encourage you to share with your dedicated Biogazelle project manager any information about the selected collection tubes and the protocol (e.g. manual, standard operating procedure, publication).
- Never use heparin-containing tubes for sample collection or heparin in any of the processing steps since heparin is a known inhibitor of downstream enzymatic steps such as cDNA synthesis or PCR.
- Standardize the time between sample collection and sample processing.

### Serum

- Collect whole blood in a primary blood collection tube with clot activator and without anticoagulants such as EDTA or citrate. Use for example the S-Monovette Serum-Gel 9 ml tubes from **Sarstedt** (C/N 02.1388.001).
- Allow the whole blood to clot according to the instructions of the tube provider.
- Finally, remove the clotted fraction by centrifugation at 1000–2000 x g for 10–20 minutes at 4 °C. Aliquot the upper phase (i.e. serum) and store at -80 °C.
- Use 0.25 ml aliquots for RNA extraction and aliquots between 1 ml and 5 ml for extraction of cell-free DNA.
- In general, plasma is preferred over serum, as during coagulation, nucleic acids may be released in solution.

### Plasma

- Collect whole blood in a primary blood collection tube with anticoagulants such as EDTA or citrate. Use for example the S-Monovette 9 ml K3 EDTA tubes, **Sarstedt** (C/N 02.1066.001) or the Cell-free DNA or RNA BCT tubes from **Streck** (C/N 218996 or 230248). The former

should be processed within 2 hours after blood collection; the latter allow more flexibility in plasma preparation.

- For preparation of different plasma fractions, we recommend the following procedure using EDTA tubes:
  - Centrifugation at  $\sim 120 \times g$  for 20 min at room temperature to prepare platelet-rich plasma (PRP).
  - Remove and split (e.g. 50:50) the upper liquid phase (i.e. PRP) without disturbing the buffy coat layer; aliquot (min 250  $\mu$ l) one split part and store at  $-80^\circ\text{C}$ .
  - Centrifuge the second part of the supernatant at  $\sim 1500 \times g$  for 20 min at room temperature to collect platelet-poor plasma (PPP) and a platelet cell pellet.
  - Remove the upper liquid phase (i.e. PPP) without disturbing the platelet cell pellet; aliquot (min 250  $\mu$ l) and store at  $-80^\circ\text{C}$ .
  - The platelet pellet can be collected by removing the remaining volume above the pellet and resuspension in appropriate protection solution (e.g. RNAprotect Cell Reagent); store at  $-80^\circ\text{C}$ .
- For mRNA analysis, we recommend to collect PPP. For miRNA and cfDNA analysis, all types of plasma generally yield good results.
- Use 0.25 ml aliquots for RNA extraction and aliquots between 1 ml and 5 ml for extraction of cell-free DNA.

## Urine

- Process urine samples within 2 hours after collection.
- Collect approximately 50 ml midstream urine in an empty sterile nuclease-free urine collection cup and transfer the urine to a sterile nuclease-free 50 ml tube.
- Centrifuge the tubes at  $500 \times g$  for 10 minutes at room temperature.
- Transfer the upper phase of the urine sample to a fresh sterile nuclease-free 50 ml centrifuge tube. Avoid to touch any material at the bottom of the tube. If cell pellet is of interest, please wash with PBS and flash freeze or use RNAprotect Cell Reagent from Qiagen.
- Centrifuge tubes containing the urine sample again at  $2000 \times g$  for 10 minutes at room temperature.
- Transfer approximately 42 ml of the urine, avoiding any material at the bottom of the tube, to 2 fresh sterile nuclease-free 50 ml tubes, dispensing 21 ml urine per tube.
- Store the urine aliquots at  $-80^\circ\text{C}$ .

### 3 Recommendations for nucleic acid extraction

Biogazelle uses validated, ISO17025- and GCLP-compliant procedures for nucleic acid extraction from liquid biopsies. These procedures have been carefully selected to suit the downstream applications offered by Biogazelle.

Should you prefer to perform nucleic acid extraction at your site, we recommend to take following considerations into account:

1. **Use of a suited and validated extraction method.**

Several commercial kits for nucleic acid extraction from body fluids are available. However, we recommend to use following extraction methods as they have been extensively tested and used in Biogazelle's downstream applications: miRNeasy serum/plasma kit from Qiagen for RNA extraction and the QiaAmp circulating nucleic acid kit from Qiagen for DNA extraction.

2. **Use a sufficiently large volume for extraction of nucleic acids.**

The volume of liquid biopsy used for nucleic acid extraction will directly impact the yield. Therefore, increasing the liquid biopsy input volume is advised if large amounts of nucleic acid are required. The applications at Biogazelle are optimized to reach maximum sensitivity with low RNA or DNA input amounts. Therefore, we recommend to extract RNA from 0.2 ml and DNA from 2 mL of serum or plasma. For specific instructions on other body fluids, contact to the experts at Biogazelle.

3. **Don't waste your time on integrity and concentration.**

Nucleic acid extraction from liquid biopsies typically results in low amounts of fragmented RNA or DNA. As such RNA/DNA concentration measurements are generally not reliable, we standardize our workflows based on volume input. Furthermore, we have optimized our applications to reach maximum sensitivity on minute amounts of fragmented nucleic acids. For the reasons mentioned higher, the concentration and integrity of RNA or DNA extracted from liquid biopsies should not be assessed prior to analysis at Biogazelle.

4. **Remove traces of contaminating gDNA in your RNA samples.**

Cell-free RNA may contain traces of genomic DNA that could interfere with some downstream analyses. Therefore, we recommend to perform a DNase treatment on purified RNA, if required by the downstream application. Reach out to the experts at Biogazelle to learn which applications require removal of gDNA.

## 4 Sending your samples to Biogazelle

Upon careful sample collection according to standard operating procedures and following a suitable experiment design, your samples are ready for shipment to Biogazelle. To preserve the quality of your samples, we recommend to take following considerations into account when preparing your shipment:

### General instructions

- For shipment from non-European countries we recommend to ship samples only on Monday morning. For shipment from European countries, samples can be shipped until Tuesday evening. Within Belgium, samples can be shipped until Wednesday morning.
- Please provide us the courier tracking number upon shipment.
- Provide samples in 1.5/2.0 ml tubes or 96/384-well plates except if specific requirements are listed above.
- Seal the individual sample tubes properly, preferably by means of screw caps. When using snap-lock tubes, please use additional parafilm for proper closure
- Label the individual sample tubes with a clear identifier. Do not write the sample identifier directly on the tube wall or lid but use a frost-proof sticker or label.
- Ensure that sample tubes or plates cannot be crushed during shipment.
- For dry ice shipments, ship in an insulated container (e.g. EPS with wall thickness of at least 4 cm) with enough dry ice for 3-5 days (a minimum of 10 kg is advised). Appropriate package notices must be included for dry ice shipping and shipment of known or potential biohazards.
- Contact your courier for information if you are uncertain about the regulations for shipping various materials, including known or potential biohazards.

### Nucleic acids

If more than 48 RNA or DNA samples will be shipped:

- Please provide samples in 96-well plates at uniform concentrations.
- Please ensure randomization of samples and sample groups within and across sample plates, e.g. samples from one group are provided in one 96-well plate at random positions instead of consecutive positions, or, if more than 96 samples will be shipped, they should be randomized across multiple 96-well plates.
- The sample submission form should reflect the order of the samples in the plate (i.e. samples numbered from 1 to 12 in the sample submission form are in row 1 of the 96-well plate; samples numbered from 13 to 24 are in row 2, etc.).
- Seal the 96-well plate(s) with a proper seal for storage at temperatures below -20 °C. This prevents evaporation of the samples during thawing.