CRI_Sample submission instructions and guidelines

CRI 002 (version 9)

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1 Sample collection guidelines

Irrespective of the sample type or nucleic acids isolation method, always follow these guidelines as per Biogazelle's internal process:

- **Minimize confounding variables and noise**: Eliminate variances in materials, procedures, equipment, and personnel. If elimination is not feasible, ensure these "noise" factors are acknowledged in the contract and built into your experimental design. Examples include using a single lot of isolation reagents and materials, standardization of all labware, and ensuring batch, operator or time point are characterized and minimized. In addition, samples should be collected under similar conditions and where possible at the same time, especially when different time points (days) from the same patient are included.

- **Ensure you will have sufficient samples to statistically support your conclusion (both for discovery projects and for validation)**: 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 assess this variability and provide an educated estimate of the number of samples required. An absolute minimum of three biological replicates per group is required 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 is 'the more samples per group, the better'. As such, a typical starting number when working with human individuals, is 30 samples per group.
- We recommend **validating each sample prelevation method**. Conduct a study of representative samples with your prelevation method to ensure it 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.
- Provide as much detail as possible on the **sample prelevation and isolation method at the time of sample submission.** If provided, this information will be reported back to you along with your experimental results so you may track isolation method performance against experimental results over time. It can also assist Biogazelle in resolving anomalous results should they occur.

2 Sample preservation and extraction guidelines

2.1 General remarks and guidelines

- RNA yield depends primarily on the tissue type and mass (or volume) but is also affected by disease status, chemical treatment, media, preservatives, and sample handling.
- RNA integrity depends on a wide range of factors encountered during handling of biological materials from collection to RNA isolation. Elimination of the activity of endogenous endo- or exonucleases is vital to preserving RNA integrity, and typically depends on treatment with proper preservative or freezing upon sample collection. A single thaw cycle of frozen tissue or cells is a major source of RNA degradation; careful measures need to be taken (e.g. preserve in RNA storage solution or lysis buffer and act quickly and/or on ice).
- RNA purity depends on the quality of the RNA isolation method used, the tissue type, and fixation method.
- There is natural variation in yield, degradation, and contaminants, both for "experimental" and "normal" samples. Yield, integrity, or purity from any experimental sample cannot be guaranteed.
- When working with biological samples, it is best to work quickly and to use a wet ice bath to ensure all the labeled tubes and containers are chilled. Prior to chilling of collection tubes, ensure that they have been labeled with the Sample ID using a permanent marker or properly rated labels for -80 °C storage.
- Sample IDs on tubes should be as short as possible to distinct samples from another, to avoid sample mix-up and long exposure under not optimal conditions (e.g. when selecting/identifying samples for processing) . Detailed information should be provided digitally.

2.2 Sample specific guidelines

All samples (including FFPE scrolls) should be provided in 1.5/2.0 ml Eppendorf tubes or 96/384 well plates except when specific requirements are listed in section '2 sample preservation and extraction guidelines'. e.g. deliver whole blood samples in PAXgene Blood RNA Tube.

2.2.1 Cells

After collection and cell counting, cell pellets should immediately be

- frozen in liquid nitrogen and stored at –80 °C, or
- resuspended in 700µl QIAzol Lysis Reagent (Qiagen) – vortex to homogenize – and stored at –80°C, or
- immersed in RNAprotect Cell Reagent (Qiagen) and stored at –80 °C, or
- processed (e.g. RNA extraction) as soon as harvested.

If RNA extraction is performed at Biogazelle, please provide the number of cells per vial: maximum 10^7 cells per vial is allowed.

2.2.2 Fresh frozen tissues

Tissue prelevated from a donor should be transferred to a pre-labeled, pre-chilled storage container. Snap-freezing in liquid nitrogen (prior to storage in container) or immersion in RNAlater RNA Stabilization Reagent (Qiagen) is recommended for preservation of expression profiles from tissue. Cutting the tissue into smaller pieces ensures thorough and rapid freezing. Frozen tissue should be maintained at -80 °C. Tissue mass should be measured and recorded prior to freezing. If RNA extraction is performed at Biogazelle, please provide the tissue mass for each sample. Please provide us samples of maximum 50 mg (estimated 3x3x3 mm) or 100 mg in case of tissues with high fat percentages.

2.2.3 Formalin-fixed, paraffin-embedded (FFPE) tissues

Standard formalin-fixation and paraffin embedding procedures always result in significant fragmentation and crosslinking of nucleic acids. To limit the extent of nucleic acid fragmentation and crosslinking, be sure to:

- Use tissue samples less than 5 mm thick to allow complete penetration by formalin
- Fixate tissue samples in 4-10% neutral-buffered formalin as quickly as possible after surgical removal
- Use a maximum fixation time of 24 hours
- Thoroughly dehydrate samples prior to embedding
- Use low-melting paraffin for embedding

If RNA extraction is performed at Biogazelle please provide us with 4 freshly cut sections of FFPE tissue, collected in 1.5 or 2.0 ml Eppendorf tubes, each with a thickness of 10 μm and a surface area of up to 250 mm^2. According to recommendations by the cancer genome atlas, we require

- a tumor content of at least 60% (determined on viable cells)
- homogeneous tumor content
- removal of excessive paraffin (e.g. by dissection); large amounts of paraffin can interfere with RNA extraction and the yield. The yield of RNA extracted from the samples depends on the percentage of the surface area that consists out of tissue.

We cannot guarantee an RNA yield of 100 ng of total RNA (see section 3.2) if these criteria are not met.

2.2.4 Whole blood samples

Blood (2.5 ml per sample) should be collected directly into PAXgene Blood RNA Tubes via butterfly or by syringe according to manufacturer instructions (available upon request). The PAXgene Blood RNA Tubes are supplied by, for example, BD (catalogue number 762165). Following collection, the tube should first be inverted vigorously 10 times or more, to ensure proper mixing of the preservative agent and then immediately placed into a -80 °C freezer.

2.2.5 Peripheral blood mononuclear cell (PBMC)

Typically, whole blood samples are collected in ready-to-use cell preparation tubes (e.g. 4 ml or 8 ml BD Vacutainer Cell Preparation tubes sodium citrate, BD, 362761), where cell separation is carried out within the same tube. Tubes contain a gel-like matrix (including a FICOLL Hypaque solution) which enables the separation of cells by density during centrifugation (1500 – 1800 x g, 20 min, RT). The mononuclear cells can be collected by pipetting the cell layer. Mononuclear cells are washed by adding approximately 3 x volume of a balanced salt solution (e.g. PBS), carefully suspended by pipetting or gentle inverting and centrifuged (100 - 200 x g, 10 min, RT). This step is repeated to ensure that any contaminating reagents and/or platelets are removed that may inhibit downstream applications.

Cell pellets or aliquots of these can be used directly for DNA/RNA extraction, stored in lysis buffer, further cultivated, or preserved by freezing in appropriate freezing media (e.g. 90% FBS/FCS, 10% DMSO; at -80 °C). See also section "2.2.1 Cells" for more instructions on preservation of cells.

2.2.6 Serum, plasma and other body fluids

Cell-free serum, plasma or other body fluids should be stored at -80 °C. For RNA extraction from serum or plasma, 200 µl sample is recommended as input amount. Please provide us with 250 µl aliquots. For extraction of cell-free DNA from serum or plasma, 1 to 2 ml of sample is advised.

In order to isolate circulating, cell-free nucleic acids from serum, plasma or other body fluids, appropriate and standardized procedures to separate fluids from cells, particles or debris should be followed, to ensure highest RNA yield and to minimize variation caused in pre-analytical steps.

The choice of collection tubes, the collection protocol, the centrifugation force (speed, duration and number of steps) and the biology of the sample have an influence on the RNA yield and the analysis/interpretation of downstream results. Therefore, information about the selected collection tubes and the protocol (e.g. manual, standard operating procedure, publication etc.) should be provided.

At Biogazelle, body fluid samples with various parameters have been successfully processed. Below, we provide recommendations.

2.2.6.1 Serum

Collection of whole blood in a primary blood collection tube (e.g. S-Monovette Serum-Gel 9 ml tubes, Sarstedt, 02.1388.001) with clot activator and without anticoagulants such as EDTA or citrate. The whole blood is allowed to clot following the instructions of the tube provider. The clotted fraction is typically removed by centrifugation at 1000–2000 x g for 10-20 minutes at 4 °C. The upper yellow phase (=serum) is aliquoted (min 250 µl) and stored at -80 °C.

2.2.6.2 Plasma

Collection of whole blood in a primary blood collection tube (e.g. S-Monovette 9 ml K3 EDTA tubes, Sarstedt, 02.1066.001) with anticoagulants such as EDTA or citrate. Blood samples intended to separate plasma should not be collected in tubes containing heparin as this anticoagulant interferes with downstream enzymatic applications, such as RT-PCR. The instructions of the tube provider are followed. The cell fraction is typically removed by centrifugation at 1000–2000 x g for 10-20 minutes at 4 °C. The

upper liquid phase (=plasma) is aliquoted (min 250 µl) and stored at -80 °C. Plasma preparation is ideally done within 2 hours of blood collection.

For a differential fraction collection (e.g. platelets), we recommend whole blood collection as above, followed by centrifugation at ~120 x g for 20 min at 4 °C to collect platelet-rich plasma (PRP). The upper liquid phase (=PRP) is aliquoted (min 250 µl) and stored at -80 °C and/or the (remaining) supernatant centrifuged at ~1500 x g for 20 min at 4 °C to collect platelet-poor plasma (PPP) and a platelet cell pellet (PL). The upper liquid phase (=PPP) is aliquoted (min 250 µl) and stored at -80 °C, the platelet pellet is stored at -80 °C (with optional addition of RNAprotect Cell Reagent, see section "2.2.1 Cells").

2.2.6.3 Urine:

Collection and processing within 2 hours and storage of aliquoted samples at -80 °C. Typically, urine is collected according to the following instructions

1. Collect approximately 50 ml midstream urine in an empty sterile RNase/DNase-free urine collection cup.
2. Transfer the urine to a sterile RNase/DNase-free 50 ml tube.
3. Centrifuge the tubes at 500 x g for 10 minutes at room temperature.
4. Transfer the upper phase of the urine sample to a fresh sterile RNase/DNase-free 50 ml centrifuge tube. Avoid 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 (see section "2.2.1 Cells").
5. Centrifuge tubes containing the urine sample again at 2000 x g for 10 minutes at room temperature.
6. Transfer approximately 42 ml of the urine, avoiding any material at the bottom of the tube, to 2 fresh sterile RNase/DNase-free 50 ml tubes, dispensing 21 ml urine per tube. Please provide us with at least one tube containing 21 ml urine, preferably with 2 tubes containing 42 ml in total.

2.3 RNA and DNA extraction methods

The **extraction methods** listed in the table below are generally recommended as they are consistent with Biogazelle's internal process. However, any commercially available kit or validated extraction method that has been shown to recover RNA/DNA that meets the criteria outlined under sample input requirements can be used.

sample type	recommended extraction method *
Total RNA, including miRNA, from human whole blood collected in PAXgene Blood RNA tubes	PAXgene blood miRNA kit
Total RNA, including miRNA, from human and animal cells and tissues	miRNeasy mini kit
Total RNA, including miRNA and other small RNA molecules, from small amounts of cultured cells and various human and animal tissues	miRNeasy micro kit
Total RNA, including miRNA, from animal and human plasma, serum and other body fluids	miRNeasy serum/plasma kit
Total RNA, including miRNA, from formalin-fixed, paraffin-embedded tissue sections	miRNeasy FFPE kit
Free circulating DNA from human plasma, serum, urine or other cell-free body fluids	QiaAmp circulating nucleic acid kit
Total DNA (genomic, viral and mitochondrial) from fresh frozen tissues	QiaAmp DNA mini kit
genomic DNA from formalin-fixed, paraffin-embedded tissues	QiaAmp FFPE tissue kit

* all kits listed are provided by Qiagen.

3 Sample input requirements

3.1 RNA requirements for all service types

- RNA purity should be assessed using a UV-VIS spectrophotometer and RNA integrity using an automated gel electrophoresis system – e.g. Bioanalyzer (Agilent), Experion (Bio-Rad) or Fragment Analyzer (Advanced Analytical Technologies) – to prove its fitness for analysis. RQI/RIN numbers are an indication for the integrity of the total RNA and should be as high as possible; at least, RNA integrity values should be as similar as possible among all samples in order to avoid introducing bias.
- The integrity from RNA extracted from formalin fixed paraffin embedded (FFPE) tissues or from body fluids should not be assessed.
- RNA samples should be free of contaminating DNA; if needed, an optional DNase treatment can be performed on purified RNA.
- Determination of the RNA concentration needs to be done with a calibrated system, preferably a Nanodrop instrument.
- The total RNA submitted should be resuspended in nuclease-free water and shipped frozen on dry ice (see Shipping Instructions). Use of RNA storage solution or elution solutions (containing less than 1 mM EDTA) are acceptable and use of these solutions should be recorded in the sample submission form. Please note that the sample should be free of additives or impurities such as ethanol, guanidinium salts, EDTA (greater than 1 mM) or phenol.
- Please provide us with the RNA QC data upon shipping of the samples (together with the sample submission form).
- All RNA will be assessed upon receipt: Biogazelle will independently assess the amount of all samples provided and the quality of a random fraction or all samples (as agreed in the contract). Should there be concern about the amount or quality of nucleic acid samples after this initial assessment, you will be contacted by Biogazelle to determine whether to proceed, replace sample(s), or discard sample(s) from the study.
- Alternatively, Biogazelle can perform RNA extraction, RNA quality control, determine the RNA concentration, perform DNase treatment or test for gDNA contamination.

3.2 RNA input amounts for discovery platforms

platform	sample types	recommended input amount	minimum concentration *	minimum volume
microRNA - qPCR	Fresh frozen and FFPE tissue, cell cultures	100 ng RNA*	25 ng/µl	4 µl
microRNA - qPCR	body fluid	NA	-	1.5 µl RNA (from 200 µl body fluid)
microRNA – small RNA sequencing	Fresh frozen and FFPE tissue, cell cultures	100 ng RNA	20 ng/µl	5 µl
microRNA – small RNA sequencing	body fluid	NA	-	1.5 µl RNA (from 200 µl body fluid)
messenger RNA – RNA sequencing	Fresh frozen tissue, cell cultures, whole blood	100 ng RNA	20 ng/µl	5 µl
total RNA – RNA sequencing	Fresh frozen tissue, cell cultures, whole blood	100 ng RNA	20 ng/µl	5 µl

* Biogazelle's input amount and concentration recommendations have been established to provide good quality results, but if these cannot be met, Biogazelle can nevertheless proceed with analyses upon customer's written acknowledgement and acceptance of potential risks.

** Please note that extra sample is needed for upfront quality control (QC) and analysis. Further, excess volume (at least 5 μl) of each sample is recommended to ensure correct laboratory work.

*** Low input protocols are available upon request.

3.3 DNA requirements for all service types

The DNA should be dissolved in nuclease-free water and shipped frozen on dry ice (see Shipping Instructions). Use of elution solutions (containing less than 1 mM EDTA) is acceptable, but use of these solutions must be recorded in the Sample Submission Form. Please note that the sample should be free of additives or impurities such as ethanol, salts, phenol, or EDTA concentrations greater than 1 mM.

4 Sample shipping instructions

- For non-European countries: please ship samples only on Monday morning. For European countries: samples can be shipped until Tuesday evening. Within Belgium, samples can be shipped until Wednesday morning.
- Please provide us the tracking number upon shipment.
- All samples (including FFPE scrolls) should be provided in 1.5/2.0 ml Eppendorf tubes or 96/384 well plates except when specific requirements are listed in section '2 sample preservation and extraction guidelines'. e.g. deliver whole blood samples in PAXgene Blood RNA Tube.
- **If you send RNA or DNA, please add an aliquot of your elution buffer**. A correct concentration can only be determined if the identical elution buffer is used as blank/control.
- The individual sample tubes should be properly sealed (preferably by means of screw caps; when using snap-lock tubes, please use additional parafilm for proper closure) and labeled. Do not write the sample identifier directly on the tube wall or lid but use a (frost proof) sticker or label.

- If large numbers of RNA or DNA samples are to be shipped (starting from 48 samples):
    - 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 are to be shipped, are provided 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.*
- Make sure that sample tubes or plates cannot be crushed during shipment.
- Frozen samples should be sent in sufficient dry ice to ensure that they remain frozen until received by Biogazelle.
- 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.
- Please ensure that your purchase order (P.O.) and Sample Submission Form have been sent to Biogazelle before shipping your samples.
- Regardless of sample receipt, your project will not be initiated until Biogazelle has received your electronic Sample Submission Form and Purchase Order.

Please use the following shipping address:

Biogazelle
Technologiepark 3
B-9052 Zwijnaarde
Belgium

5 Instructions to complete your Sample Submission Form

Please use the exact shipping address provided above when shipping your samples.

- Contact and project information:
  - Provide the name, phone number, and email address of the primary contact for the project. This enables Biogazelle to reach the responsible person quickly and efficiently if there are questions regarding the samples or project.
  - Please enter the sample shipment date.
  - Provide your Quote Number to ensure proper tracking of your project.
  - Sample storage solution information is vital to ensure proper sample processing.
- Give a brief description of your project including information relevant for the analyses to be performed by Biogazelle.
- Enter your Sample Information:
  - Enter the identifier that is affixed to each tube in your shipment package.
  - Enter the mass (if relevant), volume, concentration, and origin of the sample (organisms and tissue, cell, or fluid).
  - Provide us with relevant sample information. These may be categorical factors, such as "cell type" or "treated/untreated" or continuous values such as "dose" or "time". This sample information will be used throughout your study and conveniently reported with your results if relevant.
- The sample submission form contains a hidden worksheet. This hidden worksheet is for internal use at Biogazelle only and assures the traceability of your samples and their subsequent analysis. Please, do not make changes to this worksheet.

6 Checklist for completion of sample submission

- The correct shipping address is used: Biogazelle NV, Technologiepark 3, B-9052 Zwijnaarde, Belgium
- Quotation, including statement of work, Terms and Conditions and sample submission instructions and guidelines signed by the authorized representative and sent to your Biogazelle contact person by email
- Purchase Order that references the quote provided to your Biogazelle contact person by email
- Sample Submission Form including names and details of the submitted samples in an electronic format sent your Biogazelle contact person.