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Foreword

The Guidance Document on good practices and standardisation of sample collection for omics analysis aims to enhance the reliability and regulatory acceptance of omics data by describing essential processes required to maintain sample integrity. The guidance applies primarily to three major omics approaches: transcriptomics, proteomics, and metabolomics. Each offers mechanistic insights into biological responses to chemical exposure.

Omics technologies are increasingly being used in regulatory toxicology as part of New Approach Methodologies (NAMs). These applications include identifying molecular points of departure for risk assessment, substantiating chemical grouping hypotheses, and measuring molecular key events predictive of adverse outcomes and associative events towards mode of action determination. However, ensuring the quality and reproducibility of omics data is critical for regulatory acceptance.

The guidance emphasises best practices relevant to all omics technologies (unless stated otherwise) and to three principal sample types: in vitro (including handling adherent and suspension cell cultures, as well as media), in vivo model species (including the sampling of blood and tissues), and alternative test species (such as zebrafish embryos and Daphnia). It concentrates on the pre-analytical phase of omics studies, addressing five critical processes to ensure sample integrity: study exit, sample collection, processing, storage, and transportation. The guidance does not encompass analytical techniques beyond these five processes, such as sample extraction and omics data generation. The guidance is not intended to contain precise procedural details as would be expected from a stand-alone Standard Operating Procedure (SOP). Instead, users should use this document in conjunction with instructional manuals. However, the Annex 2 to this guidance provides several examples of relevant protocols, aiming to help disseminate good practices for omics sampling.

The guidance emphasises the importance of documentation outlining the sampling protocols, deviations from standardised processes, and metadata capture to ensure consistency and transparency. It complements the OECD Omics Reporting Framework (OORF), promoting transparency in data reporting for regulatory applications. Furthermore, it aligns with other international standards, for example, those produced by the International Organisation for Standardisation and the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use, as well as best practices from biorepository organisations.

The Guidance document was developed by an ad hoc drafting group and supported by the OECD Expert Group on Omics, with coordination from the European Chemicals Agency (ECHA). It was reviewed by the Working Party on Hazard Assessment (WPHA) and the OECD Extended Advisory Group on "Emerging Science in Chemicals Assessment" (ESCA) in April 2025. The present guidance document was reviewed by the WPHA in May 2025 and received approval by the WPHA at the June 2025 meeting. It is published under the responsibility of the OECD Chemicals and Biotechnology Committee.

Implementing the OECD guidance is expected to enhance the reliability of omics data for regulatory toxicology applications. These best practices for omics sample collection will facilitate the broader adoption

of omics technologies across chemical policy domains and jurisdictions, benefiting all OECD Member Countries and supporting the transition to NAMs.

Disclaimer: Annex 2 of the document includes detailed standard operating procedures (SOPs). These are provided, at the request of OECD Member countries, as illustrative examples of laboratory procedures associated with sample collection for omics analysis. In many cases, the SOPs include products identified by tradenames, however, the mention of a trademarked product does not indicate OECD support and other similar products may be used. In addition, the SOPs provided are not exhaustive regarding acceptable methods for sample collection, nor have the SOPs included been reviewed by OECD.

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Executive summary

Omics technologies are increasingly used in regulatory toxicology as part of New Approach Methodologies (NAMs) to identify molecular points of departure for risk assessment, substantiate chemical grouping and measure molecular key events predictive of adverse outcomes. Ensuring the quality and reproducibility of omics data is critical for regulatory acceptance. This involves maintaining sample integrity through proper handling and processing.

The guidance document on good practices and standardisation of sample collection for omics analysis aims to enhance the reliability and regulatory acceptance of omics data by describing essential processes required to maintain sample integrity. The document covers three major omics approaches: transcriptomics, proteomics, and metabolomics, each providing insights into biological responses to chemical exposure. It aligns with other international standards and best practices from biorepository organisations.

The guidance document focuses on the pre-analytical phase of omics experimentation, encompassing study exit, sample collection, processing, storage, and transportation. It provides best practices relevant to omics technologies and various sample types, with a focus on detailed procedures for sample collection, including the importance of rapid handling, maintaining consistent environmental conditions, and the use of appropriate labware. It includes examples of Standard Operating Procedures (SOPs) that aim to disseminate good practices for omics sampling, but these are not exhaustive.

General recommendations for users of this document

- Use this document in conjunction with instructional manuals
- Adhere to best practices relevant to all omics technologies and sample types, focusing on the pre-analytical phase to ensure sample integrity
- Refer to Standard Operating Procedures (SOPs) in Annex B for examples
- Maintain thorough documentation outlining sampling protocols, deviations from standardised processes, and metadata capture to ensure consistency and transparency.

Specific Recommendations:

- Study exit:
    - Records: Describe the status and/or characteristics of the biological test system at the time of sampling and keep consistent environmental conditions across all samples within a study.
- Sample Collection:
    - Quantity: Ensure that the amount of sample collected is fit for the intended purpose of the investigation, considering the omics approach(es) to be used, in consultation with the analytics provider, while at the same time preventing unnecessary collections or compromising tissue availability for other applications such as *in vivo* regulatory guideline study.

  - Timing and Consistency: Perform sample collection in a consistent manner to avoid introducing technical variability in the transcriptome, proteome, and/or metabolome.
  - Environmental Conditions: Maintain consistent environmental conditions and across all samples within a study throughout the sampling procedure.
- Sample Processing:
  - Procedures for sample processing: Wash and/or filter samples to isolate the sample type of interest and remove residual contaminants.
  - Sample quenching: Quench samples to halt enzymatic processes and metabolism and preserve biomolecular profiles.
  - Sample stabilisation and fixation: Use appropriate additives for processing certain sample types (e.g., RNA stabilisation reagents, fixatives, etc.).
- Sample Storage:
  - Temperature: Store samples at -80°C or in liquid nitrogen to maintain original composition and integrity for the period between collection/processing and the analytical phase.
  - Freeze-Thaw Cycles: Minimise freeze-thaw cycles to prevent samples degradation.
- Sample Transportation:
  - Packaging: Use appropriate packaging to prevent damage and maintain environmental conditions.
  - Temperature: Ensure consistent temperatures during transportation to maintain sample integrity for omics analysis.

To support users in developing a customised protocol that maximises omics data quality for their specific test systems (e.g. sampling cells and media from in vitro systems, sampling blood and tissue from in vivo systems, and sampling whole organism alternative test species) and downstream analytical approaches, recommended procedures, specific considerations, and reporting guidance are provided for the five pre-analytical processes. These procedures can be found in Sections 4, 5, and 6. Finally, there is a section on documentation of sampling protocols, deviations, and metadata, which encourages reporting of sample and study conditions descriptors, including standardisation in metadata fields and naming conventions to ensure comparability across studies.

1. Introduction to OECD guidance on sampling for omics

1.1 Background

Omics approaches, such as transcriptomics, proteomics, and metabolomics, are increasingly being evaluated for their contributions to regulatory toxicology (Buesen *et al.*, 2017). Applying these technologies to identify and characterise hazards is considered a New Approach Methodology (NAM; (Patil, Satpute and Nalage, 2023)), which has significant potential for replacing, refining, and reducing animal testing. Principal regulatory applications include (1) identifying molecular points of departure that are predictive of reference doses derived for apical endpoints from traditional toxicology studies (Farmahin *et al.*, 2017), (2) generating bioactivity data that provide weight of evidence towards substantiating grouping hypotheses required for read-across (Viant *et al.*, 2024b), (3) deriving plausible toxicological interpretations based on mechanistic data by measuring molecular key events that are predictive of adverse outcomes and associated events towards mode of action (MoA) determination and development of adverse outcome pathways (AOP; (Bajard *et al.*, 2023)). Table 1 provides several examples of the potential applications of omics in regulatory toxicology.

Table 1. A set of example references provided by experts demonstrating the types of omics technologies and their various applications to: (1) derive molecular points of departure (PoD), (2) justify or derive a grouping hypothesis (G), or (3) interpret molecular effects to derive support for a mode of action (MoA). Omics technologies covered here include transcriptomics (Tx), proteomics (Px) and metabolomics (Mx). The complete list of expert-suggested literature is available in Annex 1.

Example Reference	Omics technology			Application			Test system
	Tx	Px	Mx	PoD	G	MoA	
(Bundy *et al.*, 2024)	X			X			*in vitro*
(Bannuscher *et al.*, 2020)		X	X		X		
(Malinowska *et al.*, 2023)			X	X			
(Marable *et al.*, 2022)	X		X			X	
(Rempel *et al.*, 2015)	X				X	X	
(Canzler *et al.*, 2025)	X	X	X			X	*in vivo*
(Mezencev and Auerbach, 2021)	X					X	
(Viant *et al.*, 2024a)			X		X		
(Bhat *et al.*, 2013)	X			X		X	
(Page-Lariviere, Crump and O'Brien, 2019)	X			X			
(Karkossa *et al.*, 2021)		X	X			X	*in vitro & in vivo*
(Moffat *et al.*, 2015)	X			X		X	
(Villeneuve *et al.*, 2024)	X			X			

(Essfeld *et al.*, 2024)	X	X				X	*Alternative test species*
(Ayobahan *et al.*, 2019)		X			X		

Adopting omics approaches in a regulatory setting requires that hazard and risk assessors can confirm that omics data are of sufficiently high quality to provide reliable conclusions. An essential component of such assessments is the transparent reporting of methodologies used to acquire, process, and statistically analyse the omics data, along with the associated data and metadata. To this end, the OECD Omics Reporting Framework (OORF)(OECD, 2023a) was published to provide data submitters with a reporting template, ensuring the transparency, reliability, and repeatability of omics data when used for regulatory applications (Harrill *et al.*, 2021b), including IATA case studies (OECD, 2017a). Furthermore, the OECD is developing guidance for reporting omics studies for specific regulatory settings, such as chemical grouping for read-across (OECD, 2017b). While these recent and ongoing activities are important for promoting the regulatory acceptance of omics approaches, one of the first steps in generating sufficiently high-quality omics data for chemical risk assessment is the collection and storage of samples occurring during the first or "pre-analytical" phase of an omics study (Figure 1).

Several international organisations have developed guidelines and best practices related to sampling biological materials for omics and other molecular analyses, which multiple stakeholders from industry, government, and academia have supported. These guidelines have been consulted as part of developing this Guidance Document. For example, the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) published 'Guideline E18' on *Genomic sampling and management of genomic data*, thus providing valuable information, albeit restricted only to samples associated with deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) (EMA, 2017). The International Agency for Research on Cancer (IARC) also published guidelines for biological sample collection, processing, storage and information management, including for omics analysis (Vaught and Henderson, 2011). The International Organization for Standardization (ISO) described a detailed procedure for *Molecular in vitro diagnostic examinations — Specifications for pre-examination processes in metabolomics in urine, venous blood serum and plasma* (ISO, 2021). More broadly, best practices and managing biological materials are published by the International Society for Biological and Environmental Repositories (ISBER 2024) — *Best Practices: Recommendations for repositories* (Campbell *et al.*, 2018). Additionally, many "white papers" and reviews describing various aspects of omics sampling have been published in scientific journals (Holland *et al.*, 2003; Kirwan *et al.*, 2018; Gonzalez-Dominguez *et al.*, 2020); Chen *et al.* (2023c); (Chen *et al.*, 2023b). The current Guidance Document has been written to support multiple stakeholders, specifically for chemical risk assessment, to help ensure the adoption of good practices for collecting samples for omics analyses. This will facilitate the generation of high-quality omics data within a regulatory setting.

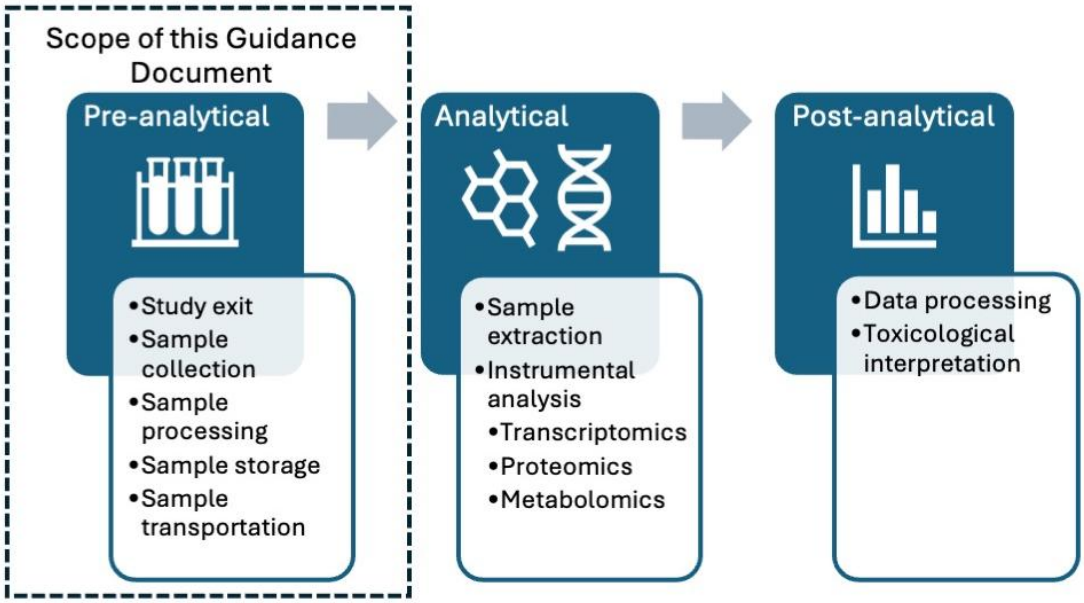

Figure 1. Overview of the three phases of an omics study and their associated processes. This OECD Guidance Document focuses on the five processes within the pre-analytical phase (indicated by the dashed line). However, users of this Guidance Document are advised to contact the analytical (omics) facilities for additional instructions, especially regarding the required material needed for sample extraction and instrument analysis, which are discussed.

1.2 Objectives of guidance

The objectives of this Guidance Document are the following:

1. To increase knowledge of the critical factors associated with five pre-analytical processes (i.e., study exit, sample collection, processing, storage, and transportation; Figure 1) to obtain a sufficiently high-quality sample for omics analysis.
2. To provide guiding principles to help increase the harmonisation of these pre-analytical processes towards standardisation, which underpin high-quality omics studies (*in vitro*, *in vivo*, and alternative test species) for applications in chemical risk assessment.
3. To disseminate good practices for omics sampling by providing examples of Standard Operating Procedures (SOPs).
4. To maximise the value of ongoing experimental studies by encouraging sample (and metadata) collection, processing and long-term storage to enable future omics measurements.
5. To ultimately promote the regulatory uptake of omics to accelerate the deployment of NAMs, especially those methods that can reduce or entirely replace traditional animal testing.

1.3 Scope of guidance

The scope of this Guidance Document is described below and illustrated within the context of a toxicology study employing omics analysis (Figure 1):

1. The guidance is intended to introduce the general principles for conducting sampling to enable omics measurements in studies using *in vitro, in vivo* and alternative test species. It only focuses on pre-analytical processes and does not describe good practices for the subsequent analytical steps in an omics study (e.g., it does not cover the extraction of biomolecules).
2. It provides sampling guidance for the most widely used omics approaches, specifically transcriptomics (which refers to bulk RNA sequencing), proteomics, and metabolomics, which includes lipidomics.
3. A wide range of sample types is considered, including *in vitro* (adherent cells, cells in suspension, cell media), *in vivo* (biofluids, such as blood, and tissues), and alternative test species (e.g., zebrafish embryos, *Danio rerio*, and *Daphnia magna*). This document focuses on sample types derived from the biological systems studied in the current OECD Test Guidelines.
4. The guiding principles apply to any sample collection for omics analyses. However, if sampling is incorporated as part of an existing OECD Test Guideline study, there may be additional practical considerations, such as those necessary to ensure the regulatory validity and outcome of that study. Experimental design, including specific recommendations for which sample types (e.g., tissues) should be collected during a regulatory study, is also outside the scope of this Guidance Document. However, a technical note on considerations for additional sampling of blood and tissue(s) as part of a regulatory study is provided in Annex 3.
5. With its primary focus on providing guidance on good practice rather than the reporting of omics sampling, this Guidance Document should be used in conjunction with the OECD Omics Reporting Framework (OORF (OECD, 2023a)), which provides reporting elements for the regulatory use of omics data from laboratory-based toxicology studies or equivalent documentation.
6. The recommendations within this Guidance Document are principles only. They should be interpreted in accordance with the legislations, regulations, and policies applicable in each jurisdiction where the omics studies are conducted.

1.4 Structure of this document

To meet the objectives defined above, this Guidance Document is structured into seven main sections.

Section 1 introduces the use of omics in regulatory toxicology as well as the objectives, scope and structure of this OECD Guidance Document on Good Practices and Standardisation of Sample Collection for Omics Analysis.

Section 2 begins by introducing the three main types of biomolecules studied using omics approaches (transcriptomics, proteomics, metabolomics), their properties, and the resulting implications for sampling to ensure the integrity of these biomolecules before their analytical measurement.

Section 3 introduces the five pre-analytical processes used in sampling for omics approaches, which comprise study exit, sample collection, sample processing, sample storage, and sample transportation, along with definitions of these terms. General procedures applicable to most sample types measured using any of the three omics approaches are then presented.

Sections 4-6 introduce the range of sample types that are routinely measured using omics approaches, comprising *in vitro* (Section 4), *in vivo* (Section 5), and alternative test species (Section 6). Within each section, the individual pre-analytical processes from study exit, sample collection, processing and storage to sample transportation are described while highlighting test-system-specific considerations. Storage and sample transportation are combined because the considerations for these processes are related. The sections provide the reader with example sampling procedures to highlight key points. Should a particular omics approach require unique considerations given the nature of the biomolecules being measured, this is highlighted under the relevant processes.

Section 7 describes the recommended metadata capture and standardised reporting related to sampling for omics, extracted from the OECD Omics Reporting Framework (OORF; (OECD, 2023a)), illustrated with examples.

2 Introduction to omics technologies, properties of biomolecules and implications for sampling

2.1 Introduction to omics analysis

There are more than 100,000 biomolecules present in a cell that participate in biological processes (Veenstra, 2021). The term “omics” describes a variety of modern approaches used in biology that attempt to comprehensively measure a particular class of biomolecules in terms of their identity, form, function and/or quantity (Narad and Kirthanashri, 2018). Approaches include (1) genomics, which measures nucleic acids arranged as deoxyribonucleic acids (DNA) within the cell nucleus and the mitochondria, providing genetic information; (2) epigenomics, which detects molecular changes contributing to altering gene expression; (3) transcriptomics, which identifies and quantifies all messenger ribonucleic acids (mRNA) expressed in a system; (4) proteomics, which identifies and quantifies all proteins and their post-translationally modified forms in a system; and (5) metabolomics, which measures low molecular weight (typically <1,500 Da) biochemicals (metabolites) in a system. This Guidance Document concerns explicitly the collection of samples for the latter three approaches: transcriptomics, proteomics and metabolomics. However, the guidance on sampling for transcriptomics is equally applicable to genomics.

Each of these omics technologies can provide unique mechanistic information about a biological or toxicological process (Buesen *et al.*, 2017). Integrating metabolomic and transcriptomic data enables a more robust interpretation of toxicological responses by linking changes in gene expression with altered biochemical pathways and uncovering the underlying mechanisms of action. Because many metabolomic changes occur downstream of gene expression, triangulating these two complementary data types can help reveal how altered transcriptional programs translate into functional biochemical outcomes.

Among the three omics technologies considered here, changes in the transcriptome will form a large component of the earliest functional response (or bioactivity) to chemical exposure. A substantial fraction of metabolite changes occurs downstream, providing mechanistic insights into the causal links towards the eventual adverse outcome. Proteomic changes bridge transcriptional responses and metabolomic shifts, reflecting both pathway activation and post-translational modifications critical for toxicological responses. However, the response time within and among omics layers varies substantially, and the biological flow of information is not strictly linear but instead consists of complex, interconnected regulatory networks with distinct temporal scales (Canzler *et al.*, 2020). The order in which the omics technologies are described in this Guidance Document – transcriptomics, proteomics, and then metabolomics – should not be interpreted as a strict causal sequence (Figure 2). When combined, omics datasets can also provide richer and more informative insights into time-dependent dynamics, pathway interactions, and compensatory mechanisms, rather than just early or downstream responses (Table 1). Multi-omics integration approaches help unravel these dynamic interactions and should be incorporated in study designs where feasible.

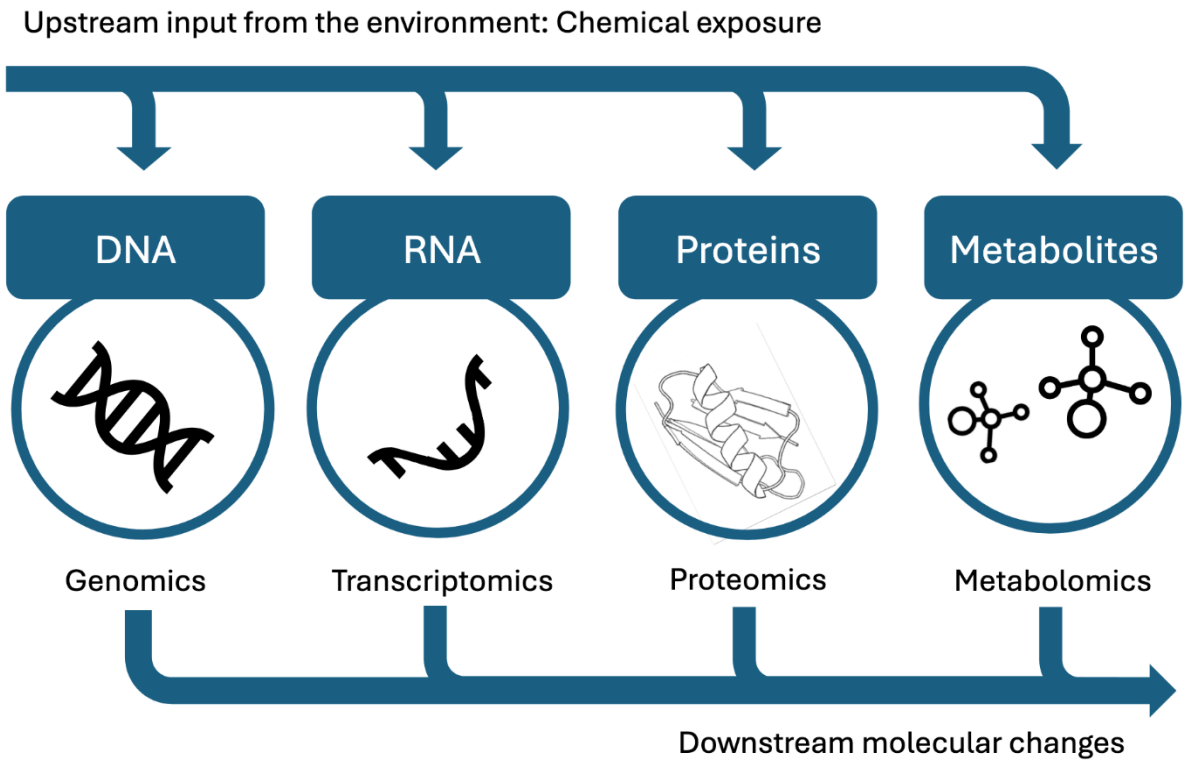

Figure 2. Representation of the central dogma of molecular biology. The flow of genetically encoded information to produce functional biomolecules is unidirectional. However, regulatory feedback, post-translational modifications and metabolite-driven influences create interconnected molecular networks that modulate this process. The influence of environmental conditions (including chemical exposure) can act at multiple levels, leading to dynamic molecular responses that are detectable via omics technologies.

Omics technologies are capable of highly sensitive measurements to identify and quantify biomolecules within a sample, providing insights into how the biological system responds to chemical exposure (Table 1). To generate relevant and reliable information, the biomolecular profile of a given sample at the point of omics measurement should ideally reflect the true biomolecular profile within the biological system under investigation, i.e., the omics profile should qualitatively and quantitatively represent the underlying biology and molecular processes in the sample at the time of collection.

This can be achieved by carefully selecting and implementing a series of pre-analytical processes from study exit, sample collection, processing and storage to (often) transportation to a specialist laboratory for omics analysis. In the section below, transcriptomics, proteomics and metabolomics technologies are briefly introduced to provide background information for a reader new to this field. The underlying properties of RNA, proteins and metabolites are then considered, specifically from the perspective of how biomolecular properties can influence the sampling procedures needed to achieve high-quality omics measurements.

2.1.1 Transcriptomics

Transcriptomics is also referred to as gene expression profiling (Chen *et al.*, 2023a). It is the systematic study of an organism's transcriptome, achieved by quantifying the sum and diversity of all ribonucleic acids (RNA) produced by cells. The transcriptome is composed of transient (intermediary) molecules in a genome-wide regulatory network. Messenger RNAs (mRNAs) are subsequently translated into proteins, while non-coding RNAs, such as microRNAs, act as RNA modulators and are particularly relevant for fine-

tuning the gene expression of eukaryotic cells. Transcriptomes are highly dynamic and can actively change by the magnitude of the gene expression and by alternating their isoforms (i.e., through alternative splicing). Transcriptomes are modulated by many factors, including exposure to xenobiotics, cell type and tissue, epigenetic modifications, hormonal signals, and circadian rhythms. They can signal gene regulatory responses to any experimental treatment affecting a cell's or organism's biology.

There are primarily two primary methods to study transcriptomes comprehensively (Lowe *et al.*, 2017). Microarrays use a hybridisation-based approach, allowing for relative quantification of gene expression. RNA sequencing (RNA-Seq), a next-generation sequencing method, sequences cDNA fragments generated from RNA molecules. RNA-Seq provides a more comprehensive and sensitive quantification of transcript abundance by counting the number of sequencing reads aligned to a gene. Targeted RNA-Seq is also an option for gene expression analysis of a focused set of genes of interest by either enrichment or amplicon-based approaches (Hrdlickova, Toloue and Tian, 2017).

Overall, to obtain an accurate representation of the transcriptome, processes within the pre-analytical phase should minimise unintentional alterations in the abundance and/or integrity of RNA. Protocols on sample collection for subsequent transcriptomics analysis should consider the susceptibility of RNA to largely RNase-mediated degradation amongst other factors as introduced below.

2.1.2 Proteomics

Proteomics focuses on the comprehensive analysis of all proteins and endogenous peptides within a biological system (Chen et al., 2023a). It involves identifying, characterising and quantifying proteins and their post-translational modifications (PTMs). These analyses provide insights into the functions and interactions of proteins in various biological processes. Proteomics also helps understand how organisms respond to different environmental conditions, including chemical exposure. Proteins are biomolecules composed of polypeptide chains consisting of amino acids that can fold into intricate three-dimensional structures. Many biological functions, including structure maintenance, metabolism and cell signalling, are enacted through proteins, thus making them crucial determinants of cellular and organism phenotypes, as well as important targets of chemical actions.

There are primarily two main methods to comprehensively study proteomes (Cui, Cheng and Zhang, 2022), while innovations are steadily improving throughput (Haslam et al., 2022). In the bottom-up approach, proteins are first digested into predictable peptides using enzymes such as trypsin. Then the peptides are separated and analysed, typically using liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). In the top-down approach, intact proteins are analysed without prior digestion.

Overall, to accurately capture the target proteome and peptidome whether through targeted or untargeted analysis and to minimise pre-analytical alterations, procedures should aim to preserve proteins and endogenous peptides in their native, unmodified state. In designing sampling protocols, it is crucial to understand and adequately control all the factors that can jeopardise protein integrity and, hence, the accuracy of subsequent proteomics analysis.

2.1.3 Metabolomics

Metabolomics is the systematic study of the levels of small molecule endogenous metabolites (i.e., of low molecular weight, typically <1500 Da) within cells, tissues or whole organisms (Chen *et al.*, 2023a). In the context of this guidance, the term 'metabolomics' includes the measurement of both endogenous polar and non-polar compounds. Therefore, this term includes the field often referred to as 'lipidomics'. Metabolomics measurements can provide information on both early and downstream functional molecular responses to chemical exposure. Metabolites are produced and consumed in biochemical reactions catalysed by enzymes (proteins). These biological reactions can be rapid and lead to the depletion or increase in metabolite levels on timescales as short as seconds or minutes.

Metabolomics measurements can be either targeted or untargeted and are typically conducted using high-resolution mass spectrometry and, to a lesser extent, by nuclear magnetic resonance (NMR) spectroscopy (Chen *et al.*, 2023a). Separation technologies (e.g., liquid chromatography or gas chromatography) are often employed to resolve the complex mixture of metabolites and lipids prior to analysis by mass spectrometry. Due to the high analytical sensitivity and selectivity of liquid chromatography-mass spectrometry (LC-MS), when operated in an untargeted mode, this approach can often detect endogenous metabolites as well as the exposure chemical(s) and/or its biotransformation product(s). It can thereby provide information on the biological response to chemical exposure (i.e., bioactivity) and the fate of the exposure chemical simultaneously (Bowen *et al.*, 2023).

Overall, to accurately measure the target metabolome, processes within the pre-analytical phase should focus on immediately stopping (e.g., rapidly immersing in liquid nitrogen within seconds, when possible) or strongly inhibiting the normal enzymatic biochemical reactions occurring within a biological sample. Furthermore, due to the high sensitivity of mass spectrometry for measuring low molecular weight chemicals, significant attention should focus on avoiding, or minimising, any chemical contamination of samples during the pre-analytical stage as discussed below.

2.2 Properties of biomolecules and implications for sampling

The biomolecular profiles are particularly susceptible to change rapidly or degrade due largely to enzymatic processes and may also be affected by contamination from a variety of sources. This does apply generally for all molecular measurements and specifically for those that are measured using omics technologies. There are two primary types of unwanted changes that can occur prior to their measurement that may introduce experimental bias and variation, thus affecting the relative abundance and/or integrity of biomolecules: (1) those that change molecular concentrations following a definable pattern, e.g., time elapsed after collection, and (2) random or stochastic concentration changes resulting in sample-specific technical variation, e.g., a freeze-thaw cycle of some or all samples within a study (Chen *et al.*, 2023a; Chen *et al.*, 2023b). Such pre-analytical sampling inconsistencies and errors will likely result in the collection of omics data that are not truly reflective of the biomolecular profile within the sample at the point of collection, which cannot be easily compensated for (e.g., through advanced instrumentation for measurement or statistical analysis). Hence, these inadequacies should be minimised or avoided entirely through careful planning and strict adherence to SOPs that describe the sampling process to maintain the stability and form of the biomolecules of interest [ISBER, Best Practices: Recommendations for Repositories (Snapes *et al.*, 2023)]. Implementing SOPs for sample collection, processing, and storage is essential to help maintain uniformity of the procedures. Documenting all steps in the sampling process is also crucial as it facilitates data interpretation and comparison among samples, which is discussed later in Section 7 of this guidance.

This section introduces a series of properties of biomolecules that significantly impact sampling for one or more types of omics analysis, as illustrated by examples in Table 2. Detailed considerations for sample collection based on these biomolecular properties are provided in Section 4 (*in vitro* test system), Section 5 (*in vivo* test system), and Section 6 (alternative test species). This guidance also highlights if one type of omics is particularly susceptible to unwanted biomolecular changes.

2.2.1 Endogenous biochemical reactions can rapidly change biomolecular profiles during and after sampling

All three types of biomolecules have varying half-lives by naturally occurring cellular biochemistry, with some existing for very short periods of time (Schwanhäusser *et al.*, 2011). For example, metabolites can be particularly short-lived (on a time scale of seconds, e.g., the dephosphorylation of phosphocreatine in cardiac muscle cells) due to their metabolism by enzymes as part of normal endogenous biochemical

reactions occurring within cells. Non-enzymatic reactions (e.g., reactions among metabolites) can also lead to changes in biomolecular profiles; however, the implication of these unwanted changes is the same as for enzymatic reactions (ISO, 2021). RNA molecules are also transient, with many forms only being produced in short bursts in response to environmental conditions; the average half-life of RNA is generally accepted to be between 10-25 minutes (Wada and Becskei, 2017). Spontaneous auto-hydrolysis of the hydroxyl group in RNA molecules may also occur without the presence of enzymatic activity. In contrast, proteins tend to exhibit lower turnover than RNA, yet they can still undergo post-translational modifications (Schwanhäusser *et al.*, 2011). For these reasons, it is useful to add protease and phosphatase inhibitors to proteomics samples (Table 2, scoring).

These typical biochemical reactions can continue during and after sampling, potentially altering the biomolecular profile such that the sampled (and ultimately measured) material has a different profile from the original specimen. To minimise the impact of endogenous biochemical reactions rapidly altering the biomolecular profiles, the timing and consistency of sample collection is critical. To minimise technical variability in the transcriptome, proteome, and/or metabolome, users should aim to conduct sampling as rapidly as possible and in a consistent manner across all samples in a study. Quenching samples, e.g., by immediately flash-freezing (snap freezing) and maintaining samples at a consistently low temperature (e.g., –80 °C), also minimises the adverse impacts of enzymatic and non-enzymatic reactions that alter the biomolecular profiles.

2.2.2 Susceptibility of RNA and proteins to hydrolytic enzymes can rapidly degrade their biomolecular profiles

RNA, proteins and lipids are particularly susceptible to degradation within biological samples (Table 2, scoring) due to potential endogenous and exogenous (e.g., due to microorganisms) hydrolytic enzymatic activities (Tatosyan, Ustyantsev and Kramerov, 2020). Due to the high potential for sample degradation, this particular type of endogenous biochemical reaction is highlighted here and described separately from the section above. These enzymes can rapidly degrade the biomolecules intended to be measured, greatly reducing the value of samples and the quality of the omics data produced. Ribonuclease enzymes (RNases) that degrade RNA are ubiquitous and difficult to remove from samples, and proteases may lead to the formation of smaller random peptide fragments. Likewise, phosphatases can remove phosphate groups from phosphoproteins, misrepresenting this important class of biomolecules. Furthermore, the single-stranded nature of RNA, coupled with the highly reactive hydroxyl group, endows RNA molecules with reduced stability compared with double-stranded nucleotides. Also, endogenous lipases can rapidly alter phospholipids within minutes if not properly managed, which can compromise the accuracy of lipidomics data.

These properties give rise to the need to quench the activity of hydrolytic enzymes, e.g., by immediately flash-freezing and maintaining samples at a consistently low temperature during storage and transportation and potentially requiring the addition of stabilising/preservative reagents to inhibit hydrolytic enzyme activity. For RNA, freeze-thaw cycles also increase the likelihood of its degradation by RNases and should be avoided (Wilfinger and Mackey, 2015).

2.2.3 Susceptibility to external conditions can rapidly degrade biomolecular profiles

The abundance and nature of RNA, proteins, and metabolites may be equally altered (Table 2, scoring) by multiple factors in the sampling environment throughout the pre-analytical phase (Thachil *et al.*, 2024). Physical factors such as temperature and air exposure may significantly produce unreliable measurements. Biomolecules may also be affected by (non-enzymatic) chemical degradation, e.g., oxidation of metabolites through exposure to air or reactions between metabolites (ISO, 2021). Furthermore, the structure of RNA may be altered by exposure to ultraviolet radiation (Snapes *et al.*, 2023). Steps should be taken to minimise exposure to external conditions that may affect a biomolecular profile

and ensure all samples within a study are exposed to consistent environmental conditions throughout the pre-analytical phase (including storage and transportation) to preserve integrity and minimise sample-to-sample variability.

2.2.4 Susceptibility to biological contaminants can alter biomolecular profiles

Biological contaminants in the context of omics sampling concern biomolecules that are either naturally present in the test system or accidently introduced during the sampling process (Hasin, Seldin and Lusis, 2017; Mills *et al.*, 2022). Examples of naturally present biological contaminants include residual cell media contaminating an omics profile if the intended sample type is the cultured cells; adjacent tissues to the target tissue that are inadvertently sampled during dissection; and food within the digestive system if the intended target is solely an alternative test species (e.g., algal feed within *Daphnia*). Accidental biological contaminants typically originate from the sampling environment (e.g., exogenous proteins or RNA from the user, or microorganisms that may be present on labware).

Contaminants can bias omics sampling and analyses (see Table 2, scoring) by introducing background signals and complicating data interpretation in addition to potentially interfering with pre-analytical and analytical processes. During sampling, it is essential to minimise biological contamination, e.g., employing sterile techniques and maintaining clean instruments and equipment. Furthermore, dedicated processes may be introduced into an SOP to reduce biological contamination, such as washing adherent cells to remove media.

2.2.5 Susceptibility to chemical contaminants can alter biomolecular profiles

Chemical contaminants are non-biological molecules that may be introduced during sample collection that can confound the biomolecular profiles of the target sample (Vuckovic, 2012). This includes substances that are deliberately added during sampling for a defined purpose that may carry additional impurities or negatively impact downstream analytical processes. For example, anaesthetics used in *in vivo* studies as well as anticoagulants added to blood (such as EDTA) may be detectable within a metabolomics profile, complicating that type of dataset and requiring removal via specialised data processing steps. Another source of chemical contaminants that can alter a biomolecular profile are those that are accidentally introduced during the sampling process (e.g., plasticisers from sampling vials and/or sample storage tubes). Moreover, certain labware can absorb certain types of biomolecules.

During sampling, it is important to record the use of any substances that are intentionally used within an SOP and to minimise the presence of other chemical contaminants that could interfere with omics analyses. Examples include using appropriate grade reagents and consumables used for sampling, selecting the appropriate labware, and ensuring compatibility of deliberate additives with downstream omics measurements.

Table 2. Summary of the potential challenges of biomolecules and subsequent considerations for omics studies, including specific considerations for individual omics. A scoring system indicates the severity of the challenge, where "+++" indicates the most severe challenge, "++" indicates a significant challenge and "+" indicates a minor challenge.

Challenges	General considerations	Specific considerations		
		RNA (transcriptomics)	Proteins (proteomics)	Metabolites (metabolomics)
Susceptible to endogenous biochemical reactions	These normally occurring reactions can rapidly change biomolecular profiles after sampling. Requires rapid handling, immediate flash-freezing (i.e., quenching), and storage below –80 °C to inhibit enzymes.	Auto-hydrolysis of hydroxyl group on RNA. +++	Maintaining optimal storage conditions can prevent post-translational modifications and normal degradation processes. +	Ongoing enzymatic reactions after sampling need to be arrested or strongly inhibited. +++
Susceptible to hydrolytic enzymes	These reactions can rapidly change biomolecular profiles after sampling. Requires rapid handling at low temperature and/or using inhibition/stabilisation reagents, and sample storage below –80 °C.	Ribonucleases (RNases) are ubiquitous and difficult to remove. Stabilisation reagents can be used to inhibit RNase. +++	Degradation by proteases and phosphatases. Inclusion of protease and phosphatase inhibitors; keeping samples on ice during collection reduce enzyme activity. +++	Lipases should be arrested or inhibited. ++
Susceptible to external conditions	Temperature and exposure to air can affect biomolecular profiles. Avoid external environments that degrade the biomolecular profile and maintain constant external conditions for all samples in a study. Biomolecules are sensitive to repeated freeze-thaw cycles. Therefore, refreezing thawed specimens for further analysis should be avoided. Specimens should be stored frozen in aliquots of appropriate quantities.	RNA is susceptible to UV light exposure, which should be avoided. ++	Proteins are susceptible to oxidation, alkylation, cross linking. Heat can denature proteins, therefore use ice or cold packs to avoid temperature fluctuations. Extreme pH levels can cause unfolding or precipitation, hence store proteins in a buffered solution at the optimal pH to minimise this effect. High salt concentrations may lead to precipitation of proteins. ++	Some metabolites are susceptible to oxidation through exposure to air. Sample storage under an inert gas can reduce this effect. Also, some metabolites degrade with visible/UV light and mild heat. Keeping samples away from direct light (e.g., amber glass vials) and storing them on ice before quenching can help minimise this effect. ++

Susceptible to biological contamination	Contaminants arising from an exposure study should be removed/reduced by appropriate processing (e.g., washing). To avoid exogenous biomolecule contamination (e.g., from microbes), work in a clean (potentially sterile) environment.	Exogenous RNA or DNA from biological contamination alters the quantification of gene transcripts by reducing its complexity during library construction or by introducing false measurements. ++	Extraneous proteins such as keratin from hair and skin can introduce significant contamination. +	Naturally present biological contaminants need to be minimised due to their impact on the biomolecular profile, such as unwanted residual cell media and dissected tissue adjacent to the target tissue. ++
Susceptible to chemical contamination	Contaminants can arise from materials used during sampling (e.g., anticoagulants, laboratory reagents) or from DNA, RNA, or protein isolation/stabilisation reagents, such as phenol, chelating agents (EDTA) and chaotropic agents (ammonium sulphate, guanidinium thiocyanate). Compatibility with downstream omics analysis should be checked.	Some reagents or components can inhibit enzymatic reactions used for transcriptomics (e.g., metal ion-chelating reagents) and must be diluted to concentrations known to be tolerated or removed through a clean-up process. +	Mass spectrometry is particularly susceptible to detecting chemical contaminants, high-purity reagents and appropriate labware should be used. ++	Mass spectrometry is particularly susceptible to detecting chemical contaminants, appropriate labware and reagents should be used as defined in an SOP. ++

2.3 Further considerations for sampling for omics

Ensuring sufficient sample quantity for omics analysis

Omics technologies can be regarded as highly sensitive, capable of detecting very low abundance biomolecules, and of detecting subtle differences among experimental conditions in biological test systems following chemical exposure. However, the precise sample quantity requirements for each omics technology differ (Misra *et al.*, 2018). While the design of an experiment is outside the scope of this Guidance Document on sampling for omics, consideration in the design should be given to ensuring that sufficient biomass is sampled to perform the intended omics or multi-omics analysis (Canzler *et al.*, 2020). For example, plate choice and well size need to be considered upfront when conducting *in vitro* experiments. Multiple analytical methods are used for each omics approach, and the specific sample requirements are dependent on the analytical platform used. Therefore, it is strongly recommended that the analytics provider is consulted. Examples of sample quantity are found in the SOPs (Annex 2), with some estimates also provided in Sections 3.3, 4.2, 5.2 and 6.2.

Adjustments for sample quantity made at the early sample collection stage are less prone to contamination and sample degradation compared to later stages. Moreover, aliquoting blood or consistently dividing the tissue into consistently smaller amounts is ideal to avoid freeze-thawing of samples, especially if multiple omics experiments are anticipated from the same sample.

Minimising systemic bias via good laboratory practice and experimental design

Due to the responsiveness of biomolecules within a biological test system to a wide range of endogenous and exogenous factors (Section 2,2), coupled with the high sensitivity of analytical omics platforms to simultaneously measure tens of thousands of biomolecules, omics studies are inherently susceptible to the following challenges: (1) unintentional effects on the biomolecular profile that are not representative of the biology immediately prior to sampling, and (2) systemic bias in the results caused by confounding variables contributing unwanted biomolecular changes, arising from the manner in which the sampling was conducted (Misevic, 2021; Timmons, Szkop and Gallagher, 2015).

For example, suppose all biological replicates within the control group were sampled earlier than the biological replicates from the chemical exposure group. In that case, the experiment may suffer from differences attributed to a temporal variable between these two groups thereby confounding the omics differences attributed to the chemical treatment. This unwanted effect may be amplified within larger studies when sampling all the biological material within one continuous period is not feasible, producing “batch effects” if groups of samples are collected at different times of the day or across multiple days. The same consideration applies to sampling from different dose groups, i.e., the duration of chemical exposure should be consistent across samples to reduce variation in treatment effects, and sampling times should be consistent across all samples.

Other potential sources of systemic bias within a study include (1) sampling by different laboratory staff (who may conduct some complex procedures slightly differently), (2) sampling under different environmental conditions (e.g., varying ambient temperature or humidity depending on locations, or fluctuations over time), (3) unintentional deviations at executing SOPs (e.g., duration of sampling steps), and (4) different lot numbers of manufactured consumables and/or reagents. These can lead to some of the sampling processes becoming “confounded” with the intended variation induced by chemical exposure.

Multiple solutions are available to avoid or at least minimise these unwanted effects to help ensure that confounding factors are not generated through the sampling procedure. SOPs should be well defined and adhered to throughout a study, with all laboratory staff undertaking the sampling processes fully trained in the SOPs. In some cases, it is appropriate to conduct a pilot study involving the stakeholder/sponsor to

confirm that all steps in an omics study are optimised. Care should be taken to ensure that the experimental design is compatible with an omics study, e.g., using randomised block designs in software such as Microsoft Excel. Block randomisation is a structured way to assign samples from different chemical treatment groups to different sampling blocks such that each treatment group is proportionally represented within a block. The order of treatments within each sampling block is chosen randomly, samples are randomly allocated to each block, and blocks are subsequently processed in random order (Burger, Vaudel and Barsnes, 2021).

To minimise systemic bias and help ensure consistency between the sampling methods and analytical objectives, detailed planning is essential. Furthermore, electronic records, e.g., describing the order of sample handling, should be taken and subsequently reported (see Section 7). Any deviations from SOPs or observations of unusual sample behaviour should be recorded in sufficient detail. Such practice may assist in identifying any mitigating factors that affect sample and ultimately omics data quality.

3 Introduction to processes used in sampling for all types of omics: definitions and general guidance

3.1 Introduction to sampling processes

The pre-analytical steps described in this Guidance Document are critical for ensuring that samples can be obtained and preserved in a manner that will subsequently allow the generation of high-quality omics data during the analytical and post-analytical phases (Figure 1.). This section describes the five pre-analytical steps of an omics study based on relevant literature and guidance.

- Study exit (Section 3.2) refers to the additional steps applied in a toxicity study that are required to prepare the test system for sampling (e.g., transfer of culture vessels from the established experimental conditions to another environment or the anaesthesia or euthanasia of a test organism).
- Sample collection (Section 3.3) refers to the process of isolating biological material(s) from a toxicity study (e.g., isolating the cells from the culture media or the sampling of blood from an organism).
- Sample processing (Section 3.4) refers to any steps required to help isolate and ensure the integrity of the biological material as it progresses through the pre-analytical phases towards omics analysis such that the biomolecular profile of the sample at the point of omics measurement reflects as closely as possible the true biomolecular profile within the biological system under investigation (e.g., separating plasma or serum from whole blood or rapidly washing and freezing adherent cells).
- Sample storage (Section 3.5) refers to maintaining samples in their original composition and integrity for the period between collection and the subsequent analytical phase including extraction and omics measurement (e.g., within a defined storage vessel in a –80 °C freezer).
- Sample transportation (Section 3.6) refers to the transfer of samples from the sampling location to an alternate location for the analytical phase, if necessary (e.g., within a defined transport vessel at low temperature using dry ice or liquid nitrogen dry shippers).

This Guidance Document is written primarily to guide the sampling of biological materials in new studies, for which laboratory staff have prior knowledge of what omics technology will be applied. In such circumstances, the most optimal sampling procedures at each step can be followed for that specific technology or combination of technologies. Each subsection below (3.2-3.6) introduces the general principles that should be considered for each of these five processes, with example procedures that are largely generic across sample types and omics technologies (Figure 3). The steps described under sample

collection and sample processing are less distinguishable for some sample types, particularly for *in vitro* samples. More detailed, specific considerations and guidance on appropriate sampling procedures for the three principal sample types are presented later in the Guidance Document in Section 4 (*in vitro* test systems), Section 5 (*in vivo* test systems), and Section 6 (alternative test species). Additionally, these sections highlight any specific conditions relevant to particular omics technologies.

Throughout the five sampling processes, laboratory staff should record appropriate metadata as well as note any unexpected observations during the experimental procedures. Section 7 outlines the recommended documentation for omics sampling in more detail with direct reference to the OECD Omics Reporting Framework (OECD, 2023a) that should be consulted in conjunction with this Guidance Document.

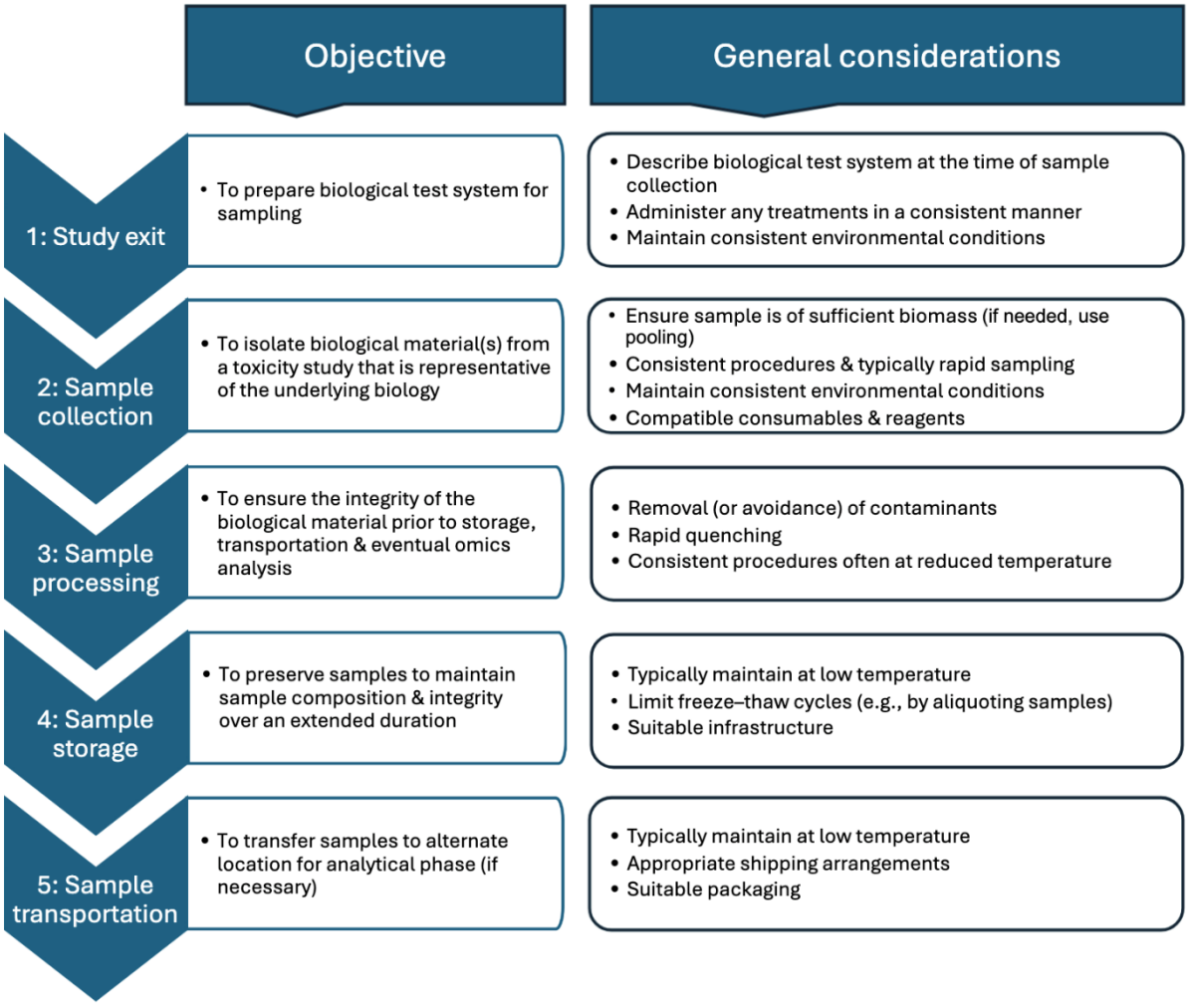

Figure 3. Main objectives and general considerations of the five pre-analytical processes used during sampling for omics analyses and largely generic across sample types and omics technologies.

3.2 Study exit

Study exit refers to the steps applied in a toxicity study that are required to prepare the biological test system for sampling. The primary purpose of study exit is to ensure that the test system is in an appropriate condition for sampling to be performed effectively and ethically.

This process may differ depending on whether the sampling is made from an on-going toxicity study (i.e., sample collection at one or multiple intermediate time points during an exposure period) or as part of the study termination. A more complex sampling process may be required for the former case to minimise any unwanted molecular perturbations that may arise from sampling, e.g., the repeated micro-sampling of blood from *in vivo* studies, where animal welfare implications must be carefully considered.

In many instances, study exit may involve the transfer of a test system to a different environmental condition (e.g., the removal of multi-well cell culture plates from an incubator to perform the sampling). A change in environmental condition can significantly influence a sample's biomolecular profile, introducing unwanted variations. Care should be taken to complete any pre-analytical procedures under consistent environmental conditions and across all samples within a study.

For *in vitro* studies, study exit is typically straightforward, requiring the removal of a cell culture plate or other culture vessel from an incubator. It may also include observation of cell morphology, although such steps would be defined in the Test Guideline. The study exit process for *in vivo* studies includes all procedures concerning the anaesthesia and/or euthanasia of test subjects, including the method, substance, dose and administration route. Additionally, observations of the test subject may be required as specified in the Test Guideline.

The study exit process for alternative test species will depend on the type of species, with some (e.g., zebrafish) using appropriate anaesthesia and/or euthanasia of the test subjects and others (e.g., the invertebrate *Daphnia*) not requiring any specialised steps.

An important aspect of study exit is to accurately record the status and/or characteristics of the biological test system at the time of sampling. This provides information that may be required to assess the reliability of the omics data obtained, i.e., by documenting possible sources of variation that may affect sample quality such as through systemic bias (Section 2.3).

3.3 Sample collection

In this Guidance Document, sample collection refers to the process of isolating biological material(s) from a toxicity study (Snapes *et al.*, 2023). The primary purpose of sample collection is to obtain material that is representative of the biomolecular state of the test system at the time of collection and is suitable for subsequent omics analysis (Figure 3).

The guidance provided here assumes the typical case that samples will be collected, processed and stored before omics analysis instead of immediately undergoing omics analysis after sample collection. There are two different sampling scenarios: (1) where the entire sample is dedicated to omics analysis or (2) where multiple measurements are made using the same sample (e.g., omics and histopathology are both applied to the same tissue or multiple omics layers should be generated from the same sample). The second scenario is more complex, and several additional factors need to be considered, such as which measurement method may have priority for the sampling and how the sample or tissue is distributed to different analyses without introducing bias (Annex 3).

Several pre-analytical variables should be considered during sample collection to ensure the integrity and suitability of samples for omics analysis. This section aims to provide guidance that mitigates potential quantity and quality issues, as addressed earlier in Table 2 and Section 2.

Quantity of sample collected

Care should be taken to ensure that the amount of sample collected (e.g., blood volume, approximate number of cells, dissected tissue weight) is fit for the intended purpose of the investigation, considering the omics approach(es) to be used while at the same time preventing unnecessary collections (e.g., in *in*

vivo studies). There are multiple analytical methods used for each omics approach, and the specific sample requirements are dependent on the analytical platform used. Therefore, it is strongly recommended that the analytics provider is consulted. Examples for sample quantity are prescribed in the SOPs (Annex 2), e.g., SOPs 2.02, 3.01, 4.02, 5.06, 6.02, 7, 8.01, 9.01 and 12.03. In an *in vivo* study, blood and tissue samples may be required for histopathology, clinical chemistry, and blood parameters, in addition to omics measurements (Annex 3). This may limit sample availability. Therefore, the priority of various analyses should be considered for the study design to not compromise the integrity of the planned analyses.

Adjustments made at this early collection stage are less prone to contamination and sample degradation compared to adjustments at later stages. When possible, the amount of sample taken should be more than the minimum requirements for the downstream analytical phase. For example, at the collection stage, the sample should be divided into multiple aliquots in the appropriate size for downstream application(s). This allows for different downstream workstreams and the potential to repeat analyses in the event of technical problems. Storing as multiple aliquots reduces manipulations of the sample that can impact its integrity, such as subjecting it to multiple freeze-thaw cycles (Ji *et al.*, 2017). However, care should be taken that the generated aliquots are homogenous with respect to their composition and physiological state (see also Section 5).

Furthermore, a balance should be found between target tissue sample size and practicality considerations (e.g., dissection time). It may not be practical, at necropsy, to collect the precise tissue sample size required for RNA isolation. In case a larger sample is collected and stored at necropsy, it may need to be cut down (while frozen) prior to extraction. While this is not ideal, if done correctly (low temperature or addition of proper preservatives), this does not substantially impact RNA quality. In general, the time for sample collection, sample processing and the number of freeze–thaw cycles should be minimised as much as possible, and highly standardised protocols should be used for all samples in a study.

Depending on the study design, planned analyses, and technologies employed, the pooling of biological test system replicates may be necessary (i.e., the combining of samples from multiple wells of a cell culture well plate; or pooling multiple zebrafish embryos, e.g., see Annex 2, SOP 12.03). However, pooling should be approached with caution, because it may complicate the conduct and evaluation of omics measurements. For example, it can obscure individual variability and amplify biases arising from differences in sample quality prior to pooling. Nevertheless, pooling samples is still necessary if there is insufficient biological material.

Procedures for sample collection

The method of sample collection can introduce significant variation between samples if it is not conducted in a consistent manner. For example, differences in the duration of collection across the samples within one study can be a significant source of variation. Consistency in the handling of biological specimens is essential alongside detailed SOPs that are strictly adhered to. This is particularly important when multiple laboratory staff and/or laboratories participate in a study to ensure that samples can be combined without introducing batch effects (EMA, 2017). If sampling for an experiment is conducted by more than one person, records of which samples were collected by which laboratory staff should be made to account for potential batch effects.

All samples should be labelled with unique identifiers, and relevant observations of the sampling procedure should be recorded throughout the study (Section 7).

The risks of chemical and biological contamination should be considered during sample collection to minimise their occurrence, e.g., microbial contamination can introduce new transcripts and metabolites into a sample, negatively impacting the derived omics profile (EMA, 2017). Good practices include the decontamination of surfaces and collection devices, use of flow hoods, and wearing appropriate personal protective equipment (e.g., nitrile gloves, laboratory coat).

Timeline for sample collection

Endogenous biochemical reactions can rapidly change biomolecular profiles during and after sampling (Section 2.2.1), and the susceptibility of some biomolecules to hydrolytic enzymes can rapidly degrade samples (Section 2.2.2). Therefore, as a general rule of thumb, sample collection should be conducted rapidly across all sample types and all omics approaches (Nakayasu *et al.*, 2021). To minimise technical variations and unintended modifications and ensure data consistency and accuracy, the timeline for sample collection should be kept consistent among all samples.

In addition, the duration of chemical exposure, e.g., as defined in the Test Guideline, should be consistent across samples to reduce variation in treatment effects (i.e., the animals that are treated first, should also be sampled first).

Biomolecular profiles may exhibit natural diurnal variation. For example, altered metabolite and hormone levels in first-morning urine have been reported when compared to collections later in the day (Kirwan *et al.*, 2018). Therefore, the time of day when the sampling is conducted should be a consideration and be kept consistent as much as possible (and recorded). This includes (1) strictly controlling the light/dark cycle in the rearing environment, (2) allowing adequate time before the experiment to allow the animals to acclimatise to the cycle, and (3) synchronising the sampling times with the light/dark cycle (e.g., administer two hours after the start of the light period). If diurnal variation cannot be avoided, a random block design should ensure that effects are distributed across groups.

Temperature during sample collection

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Types of labware used for sample collection

The instruments and appropriate collection and storage containers for samples may vary according to the sample type, downstream pre-analytical processes, and intended omics analyses. For example, RNase-/DNase-free consumables are necessary for transcriptomics applications to preserve sample integrity, and protein low-binding consumables are necessary for proteomics. At the very least, the same type of tubes (preferably from the same lot or batch) should be used within a single study.

Additionally, the integrity of sample containers and their labels may be affected by downstream processes, such as freezing in liquid nitrogen, and should be carefully selected to ensure compatibility with storage conditions (Snapes *et al.*, 2023).

Plasticisers and other substances may leach out of labware and into biological samples, which is of particular relevance to mass spectrometry-based proteomics and metabolomics. Leaching of substances from serum collection tubes has been shown to introduce interferences, including both ion suppression and enhancement, in LC-MS analysis (Chen *et al.*, 2023b). Furthermore, palmitate has been shown to leach from plasticware, obscuring the endogenous signal (Yao *et al.*, 2016). Therefore, depending on the biomolecules to be measured, labware and reagents used throughout the sample collection process should also be compatible with all downstream analytical processes, e.g., using glass vials and pipettes instead of plasticware, or pre-rinsing plastic vials and pipette tips with methanol, and also by using LC-MS grade solvents. It is recommended to incorporate process (extraction) blanks into a mass spectrometry-based omics study to detect potential contamination.

3.4 Sample processing

In this Guidance Document, sample processing includes any steps required to help isolate and ensure the integrity of the biological material as it progresses through the pre-analytical phases towards omics analysis, such that the biomolecular profile of the sample at the point of omics measurement reflects the true biomolecular profile within the biological system under investigation, e.g., rapidly washing and freezing adherent cells (Gonzalez-Dominguez *et al.*, 2020; Snapes *et al.*, 2023).

The main objective of sample processing is to help ensure the integrity of the biological material before storage, transportation and eventual omics analysis. Sometimes, sample processing is required to isolate the required sample type from other biological material.

A wide range of sample types are investigated using omics, including biological fluids (e.g., blood) and tissues from *in vivo* studies, cells and cell media from *in vitro* experiments, and alternative test species such as zebrafish embryos that are typically sampled and stored as whole organisms. This wide range of sample types has an equally wide range of complexities of the sample matrices. More complex sample types may require multiple processing steps to preserve the desired fraction (e.g., the isolation of plasma from whole blood) ensuring no cell lysis or contamination.

It is important that sample processing steps are performed consistently across all samples to prevent adversely altering the biomolecular profile or interfering with downstream analyses (Gonzalez-Dominguez *et al.*, 2020). Examples of sample processing procedures relevant to omics studies are described below.

Ideally, samples that are processed for omics analysis are flash-frozen and stored at –80 °C (Chen *et al.*, 2023c; Ahmed *et al.*, 2015).

Procedures for sample processing

Washing and/or filtering of samples may be required to isolate the sample type of interest, e.g., to remove cell culture media from adherent cells in multi-well plates, which reduces the levels of extracellular compounds, including exposure chemicals and the ingredients of the media that may otherwise act as contaminants in downstream analyses (e.g., LC-MS). The type of wash solution, the volume(s) and the washing procedure should be consistent across all samples within a given study to reduce variations.

Additional processing steps, such as centrifugation, are typically required for blood. The centrifugal parameters (including the *g*-force (centrifugal force), duration, and temperature during centrifugation applied can lead to differences in metabolite profiles and should therefore be well defined in an SOP and consistent throughout a study (Safari *et al.*, 2023).

Additives may be required for processing some sample types (e.g., stabilisers or preservatives) while other processes may include an intentional incubation on the laboratory bench, such as for clotting blood. Consistency in these steps, including the additives used, lot (or batch) number, duration and temperature of incubation, etc., is essential to avoid between-sample variation (Gonzalez-Dominguez *et al.*, 2020).

Sample quenching

In many cases, sample quenching may be required to halt enzymatic processes and prevent (or at least significantly reduce) changes to the biomolecular profiles. Quenching refers to the process of attempting to stop enzymatic activity and metabolism within a sample by rapidly lowering the temperature. Quenching of tissues that are relatively high in enzymatic activity is typically achieved by rapidly placing the excised sample into liquid nitrogen at a temperature of –196 °C (Chetwynd, Dunn and Rodriguez-Blanco, 2017). Quenching on dry ice, at a temperature of –78 °C, is not so rapid but may also be acceptable, e.g., for quenching multi-well plates from an *in vitro* toxicity study. Quenching can also be achieved using a quenching solution. For example, a commonly used quenching procedure coupled with the sample processing of cells consists of 60% methanol.

Biological samples that have relatively low or no enzymatic activity (e.g., urine samples, which have protease activity, but lower than those found in tissues) are generally less susceptible to unwanted biomolecular changes. Consequently, rapid quenching is less critical for such sample though still preferable. The specific enzymatic composition of a given sample type should be considered carefully to determine the necessity for rapid quenching. Additionally, non-enzymatic reactions are also capable of altering a metabolic profile (Keller, Piedrafita and Ralser, 2015), e.g., via oxidation (through exposure to air) and/or spontaneous reactions of reactive metabolites. Maintaining samples at a low temperature will also reduce non-enzymatic reactions.

Stabilisation of RNA

For transcriptomics analysis, RNA stabilisation reagents (also known as RNA stabilisers) can be used instead of quenching as they are designed to block RNase activity (Salehi and Najafi, 2014). These solutions may contain quaternary ammonium sulfates, caesium sulfates, guanidinium isothiocyanate and other reagents that denature RNases, DNases and proteases to prevent degradation of these biomolecules in fresh cell cultures, fresh tissue, frozen tissue, embryos, cells in blood, or whole organisms. They prevent RNA degradation from occurring and preserve RNA integrity for longer periods of time (Salehi and Najafi, 2014), even when samples are rapidly frozen, especially when using warmer temperatures for cryopreservation as freezing on dry ice or at warmer temperatures may lead to temperature gradients within the sample, leading to incomplete RNase inhibition. These reagents also prevent RNA degradation when samples are thawed for the subsequent analytical steps. Samples that are stored in RNA stabilisers are compatible with most downstream RNA extraction methods. Such samples can also be used for proteomics if additional processing clean-up steps are introduced to remove stabilisers and salts that can interfere with downstream mass spectrometry analysis (Bennike *et al.*, 2016).

However, ensuring complete penetration of the stabilising solution is critical, particularly for whole organisms or larger tissue samples, where diffusion of the stabiliser may be limited. If the reagent does not fully permeate the sample, inner tissue regions remain unprotected, leading to degradation during thawing. To prevent this, homogenisation or mechanical tissue disruption is necessary before or during stabilisation. Alternatively, a maximum recommended tissue size should be defined to ensure sufficient reagent penetration. Documentation of the stabilisation strategy, including the stabiliser used and the exact protocol followed, is essential to ensure reproducibility.

Sample fixation

Ideally, samples that are collected for omics analysis are flash-frozen and stored at –80 °C (Chen *et al.*, 2023c; Ahmed *et al.*, 2015), and therefore the focus of this guidance. However, samples may be available following fixation, primarily used for histopathology and therefore, fixation is shortly covered in this section. Additional processing steps are required when samples require fixation and are shortly covered in this section. Fixation involves chemically treating cells or tissues to modify and cross-link proteins and nucleic acids (Huang and Yeung, 2015), which stops enzymatic activity and preserves tissue morphology. Suppose preservation of tissue samples via fixation is required. In that case, paired samples may be divided for flash-freezing and fixation to allow separate samples for omics analysis and histopathology as an example. If paired samples are not possible (Annex 3), the type of fixation should be selected to ensure compatibility with downstream analyses (EMA, 2017). Common fixatives include methanol, ethanol or (para-)formaldehyde.

The feasibility of obtaining omics data from fixed samples has been primarily demonstrated for transcriptomics techniques. However, the degree of compatibility may depend upon the fixative used, the pre-analytical variables, and the storage conditions, which will be discussed in more detail in Section 3.5. There are multiple studies in which transcriptomic analysis has been performed on formalin-fixed paraffin-embedded (FFPE) tissue samples (Auerbach *et al.*, 2015; Webster *et al.*, 2015), and several commercial products and library preparation protocols like ribosomal RNA depletion (Webster *et al.*, 2015) are available. The fixation time in formalin is critical as cross-linkage of the RNA increases with time. The chemical modification of RNA such as cross-links can, however, be reduced with a heating step (Wehmas *et al.*, 2019). Several studies have demonstrated that 3-prime RNA-Seq and targeted RNA-Seq techniques are suitable for the sequencing of FFPE samples (Wehmas *et al.*, 2019; Boneva *et al.*, 2020; Trejo *et al.*, 2019).

Generally, FFPE samples produce lower-quality RNA due to higher fragmentation, nucleic acid-protein crosslinks, and the occurrence of base modifications (Babel *et al.*, 2020). Preservation procedures that subject samples to a low pH environment, like decalcification or use of Bouin's solution for preserving soft tissue morphology, may cause additional damage to nucleic acids.

Obtaining proteomics data from FFPE samples is also feasible (e.g., (Paulo *et al.*, 2012)) and further references reviewed in (Steiner *et al.*, 2014). An ISO standard published in 2018 (ISO, 2018) provides detailed recommendations for sample handling and protein isolation from FFPE-preserved human tissue samples. Metabolomic imaging of FFPE tissues using matrix-assisted laser desorption/ionisation (MALDI)-MS has been explored (Ly *et al.*, 2016).

Overall, it is recommended that the procedures for sample collection, storage and processing should be tested, optimised and standardised before a particular series of FFPE sample collection for omics analysis begins. A particular focus should be placed on verifying and validating the total RNA and protein isolation as this is the key step in the entire workflow (ISO, 2018). Fewer studies have demonstrated the feasibility of obtaining metabolomics data from FFPE samples using liquid-chromatography mass spectrometry (see citations in (Neef *et al.*, 2020)).

3.5 Sample storage

Sample storage refers to maintaining samples in their original composition and integrity for the period between collection/processing and the analytical phase (i.e., extraction and omics measurement).

Often, there is a significant period of time between a toxicity study and downstream omics measurements, e.g., all samples from a time course study are collected, or samples are collected by one laboratory before being shipped to a specialist omics laboratory or shipped to a sample archive (biorepository). As such, samples may need to be maintained with high integrity for many days, months, or even years.

Sample storage at –80 °C is the standard for maintaining the long-term integrity of biomolecules (Chen *et al.*, 2023c). An alternative is cryogenic storage using liquid nitrogen, which maintains samples below –132 °C, completely arresting biological processes, including chemical and physical reactions that may otherwise change sample integrity (Snapes *et al.*, 2023). Storage of samples using this cryogen may include submersion in the liquid phase (–196 °C) or suspension in the vapour phase (–150 °C), the latter reducing the risk of contamination and other associated hazards (Snapes *et al.*, 2023). Evidence suggests that storage of FFPE tissue samples at temperatures ≤ –20 °C may better preserve RNA (Groelz *et al.*, 2018; Schmeller *et al.*, 2019). Yet it is appropriate to store FFPE samples intended for transcriptomic analysis in a dry, dark location at ≤ 4 °C.

Freeze-thaw cycles of biological samples can reactivate enzymatic and non-enzymatic reactions and, hence, decrease the sample integrity and affect the biomolecular profile (Ji *et al.*, 2017). Where possible, freeze-thaw cycles should be avoided (e.g., by aliquoting samples prior to storage). Where freeze-thaw cycles are unavoidable, the number of cycles should be strictly minimised. Furthermore, the number of cycles should be consistent across all samples within a study and thoroughly documented (Snapes *et al.*, 2023).

Long-term sample storage requires physical infrastructure such as –80 °C freezers, ideally with back-up systems to maintain the low temperature as well as temperature monitoring and an alarm system. Storage of aliquots of samples in separate locations greatly minimises the potential loss of all samples due to equipment failure. Freezers that operate at only –20 °C are not recommended. Additionally, "frost-free" freezers which incorporate internal heating cycles should not be used as they may lead to deterioration and/or desiccation of samples stored near to the heating elements (Snapes *et al.*, 2023).

3.6 Sample transportation

Sample transportation refers to the transfer of samples from the sampling location to an alternate location for the analytical phase (if necessary).

Typically, the location of the toxicity study and sample collection (e.g., by one Contract Research Organisation, or CRO) differs from the location of extraction and omics analysis (by a second CRO), requiring samples to be transported between sites. In general, samples should not be exposed to conditions that may affect their composition and integrity during transportation (EMA, 2017).

Appropriate transport conditions should be established prior to shipment, including an anticipated timeline for sending and receiving samples (EMA, 2017; Gonzalez-Dominguez *et al.*, 2020; Snapes *et al.*, 2023). A transportation plan should consider the following factors.

Packaging of samples

Packaging should be appropriate for the number of samples and amount of refrigerant required, with samples distributed evenly throughout the vessel (Snapes *et al.*, 2023). Empty space within the container should be filled with additional packing material to prevent potential damage during shipping. The secondary containment of samples for shipping should also be considered to minimise the risk of damage.

Transportation temperature

Consistent temperatures during transportation are required to maintain sample integrity for omics analysis. Where possible, this should be equivalent to the laboratory storage temperature for the samples, discussed above (EMA, 2017).

Throughout the sample shipment, the temperature of the transport vessel should ideally be continuously recorded and documented (e.g., using a datalogger). Samples should be shipped with sufficient refrigerant

(or cryogen) to maintain the required low temperature throughout the shipping process. Should shipping delays occur (e.g., at customs), ideally the courier provides a service to replenish the refrigerant.

Several options are available for shipping samples for omics analysis and should be selected based on specific sample requirements (Snapes *et al.*, 2023):

- Ambient temperature (20-30 °C) may be suitable for FFPE samples but should still include insulating material to protect samples from extreme fluctuations.
- Ambient temperature may also be suitable for samples in RNA stabilisers; however, it is better to transport such sample types at ≤ –20 °C.
- Refrigerated transport (2-8 °C) maintained by wet ice or gel packs.
- Frozen (–20 °C) using gel packs rated for frozen temperatures.
- Frozen in dry ice (–78 °C); which is considered a hazardous material and requires specialist labelling and documentation
- Frozen in the vapour phase of liquid nitrogen (–150 °C) using a dry shipper (or vapour shipper) in which the cryogen is fully absorbed into a porous material and not considered hazardous if properly filled.

Shipping arrangements

Ideally, a just-in-time approach should be adopted, outlining an expected timeline for sending and receiving samples. Shipments should only be initiated when there are at least two working days left after the estimated delivery date to ensure samples do not arrive at weekends and/or holidays (Snapes *et al.*, 2023). Samples should be correctly classified and labelled for transportation to prevent delays and to ensure appropriate specifications are followed (Holland *et al.*, 2003).

Where possible, samples should be shipped in a manner that avoids the introduction of “shipment” as a potential confounding factor in downstream data analyses. For instance, if all exposure control samples are included in one shipment, while all chemical-exposed samples are included in a second separate shipment, then it will be impossible to equivocally attribute any biological differences between control and exposed samples solely to the effect(s) of chemical exposure. Ideally, samples should be randomised across shipping vessels (e.g., boxes) and shipments (collection-delivery cycles) to avoid this potential confounding factor.

4 Guidance on sampling cells and media from *in vitro* test systems

To increase confidence in the application of *in vitro* methods for regulatory use, the OECD has published a Guidance Document on good *in vitro* method practices (OECD, 2018). There are also several existing Test Guidelines that include *in vitro* test systems such as continuous cell lines (e.g., THP-1 human monocytic leukaemia cell line) or primary cells (e.g., human primary peripheral blood lymphocytes) (OECD, 2019; OECD, 2023b; OECD, 2024a; OECD, 2024b). In the laboratory, cell cultures are most often grown either in suspension (as single cells or clusters) or as a monolayer that is affixed to the culture vessel, reflecting the tissue type from which the cell culture was derived. For example, cells that are derived from solid organ tissues such as the liver, heart or fish gills tend to be adherent. Their differing morphology is dependent on the location (e.g., endothelial, epithelial, neural, fibroblast) within the tissue of origin. By contrast, cells derived from biological fluids such as blood tend to be cultured in suspension. This guidance document focuses on sampling adherent (2-dimensional; 2D) cells and cells in suspension. Although many sampling principles are also applicable to more complex *in vitro* test systems (e.g., organoids, organ-on-chip), there will be additional considerations for their sampling, which are not addressed in this document.

Sample collection and sample processing procedures for omics are essentially different for adherent cell cultures compared to cell cultures in suspension. Harvesting cells from adherent cultures may be performed mechanically (i.e., scraping, sonication) or enzymatically (e.g., dissociation using proteases such as trypsin) whereas cells in suspension are typically pelleted by centrifugation (Annex 2, SOP 4-6). For *in vitro* sampling, there can often be some overlap between sample collection and sample processing, which are less distinct when compared to *in vivo* test systems.

Cultured cells require a supply of nutrients for growth in a stable and sterile environment. The media composition is designed to support the growth of specific cell types and is typically defined within the OECD Test Guideline. Their constituents may contain inorganic salts, carbohydrates, amino acids, vitamins, trace elements and, importantly, fatty acids/lipids, proteins/peptides, and serum. While foetal bovine serum (FBS) is currently most used (OECD, 2018) as described in the OECD's Guidance Document on Good In Vitro Method Practices, chemically defined or xeno-free medium is preferred to maximise the reproducibility and human relevance of *in vitro* assays (Golikov *et al.*, 2022). The cellular environment must be considered when sampling cells for omics by eliminating trace media with washes, or, alternatively, conducting metabolomics investigations of the cell-free media (termed “metabolic footprinting”) to investigate metabolic interactions between the cells and their microenvironment.

The following subdocumentations detail recommended procedures, specific considerations and advised reporting for the five pre-analytical processes of sampling cells and media for omics analyses (Figure 4). Where appropriate, optional components are proposed, which are intended to help guide users in producing a customised protocol to maximise omics data quality applied to their own *in vitro* test systems and downstream analytical options. The example procedures in this section are based upon SOPs described in Annex 2 for adherent cells cultured in well plates (Malinowska *et al.*, 2023).

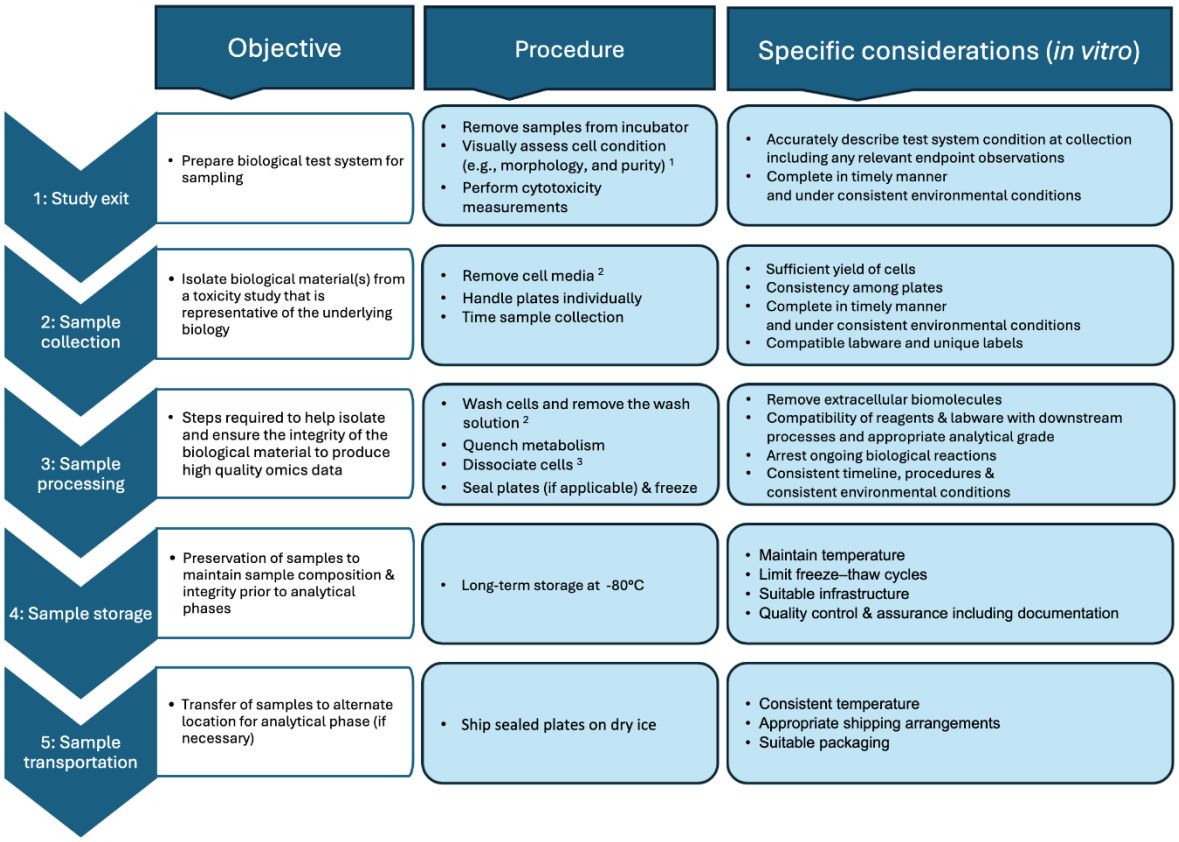

Figure 4. Main objectives, recommended procedures and specific considerations of the five pre-analytical processes used during sampling of adherent cells in multi-well plates or cells in suspension for subsequent omics analyses.
[1] Perform bright field imaging, if applicable (for quality control purposes) before sample collection and processing.
[2] For metabolomics, after removing samples from the incubator, it is often recommended to work on wet ice to slow down metabolism. For adherent cells, remove media or wash solution by pipetting, while cells in suspension require centrifugation and pipetting.
[3] Omit this step when performing high-throughput in-plate metabolite extractions.

4.1 Study exit

The study exit procedures for *in vitro* studies typically require (1) removing the culture vessel from the controlled experimental environment to a sample collection environment and (2) a microscopic assessment of the condition of the cells.

As part of the study exit of an *in vitro* study, it is often necessary to transfer culture vessels from the established experimental conditions to another environment. For example, the user may need to transfer culture vessels (such as multi-well plates) from an incubator to a cell culture hood or a benchtop to observe and record the key cell descriptors and proceed with subsequent pre-analytical steps. While sterile conditions must be maintained throughout the toxicological experiment (i.e., during cell culture and subsequent exposure of the test system to test substance(s)), adherence to sterile conditions in the subsequent steps (starting from study exit) is optional but advised whenever possible to minimise the possibility of contamination. This includes using proper laboratory procedures and equipment to avoid cell contamination (e.g., gloves). It is advised to incorporate appropriate control samples such as process (extraction) blanks / no-template controls in the toxicological experiment.

Due to the dynamic nature of the biomolecules analysed using omics technologies, a change in environmental conditions can introduce confounding factors (Section 2.2.3) and have an appreciable effect on the abundance and diversity of certain biomolecules, thereby adversely impacting downstream results (Section 2.3). Care should, therefore, be taken to complete all study exit procedures under consistent environmental conditions (e.g., temperature, duration of keeping the multi-well plates in the hood) and in a timely manner to minimise these unintentional environmental effects on the biomolecules. It is prudent to document the time when each culture vessel is removed from their experimental conditions and the time taken to complete all exit procedures to ensure a consistent timeline among samples.

Following removal of the cell culture vessel from the incubator, it is recommended to visually inspect and document the conditions of the cells (including any precipitation) using a microscope at a magnification that can detect their morphology, attachment, and degree of confluence as well as purity (i.e., lack of contamination). Omics measurements should be performed on an appropriate fraction of viable cells. At least 80% viability is recommended as a commonly used threshold although this is dependent on what cells are used and the purpose of the study. In general, the higher the fraction of viable cells, the less confounded the omics data will be through responses associated with acute stress or cell death. Therefore, it is important to perform either a concentration-range finding study first to select appropriate concentration(s) of test substance(s) for the subsequent *in vitro* omics study or cytotoxicity and/or phenotype assessments during the *in vitro* omics study itself. The latter may require performing cytotoxicity assessment in separate samples specifically dedicated for this purpose (i.e., not collected for omics analysis as reagents used for cytotoxicity assessments could interfere with omics analysis). Furthermore, to minimise cellular stress, only omics-specific steps related to study exit, collection and processing are recommended (e.g., removal of cell media and washing the cells are acceptable while an addition of cytotoxicity dye is not).

The example procedures and considerations below apply to all three types of omics (transcriptomics, proteomics and metabolomics) irrespective of the downstream analytical platform applied.

4.1.1 Recommended procedures

1. Culture vessels (e.g., multi-well plates) should be handled one at a time using proper laboratory equipment and procedures to avoid cell contamination.
2. Record the time at which the culture vessels are removed from the incubator and the duration of the exit procedures. In order to avoid timing effects, ideally the study design should reflect the time-matched exposure of e.g., controls and substance treatments.
3. Visually assess cell condition using a microscope (e.g., studies may include an additional microplate for this purpose).
4. Measure cell viability using an appropriate assay. This step may best be conducted separately using cells that are specifically devoted to these assessments (i.e., not collected for omics analysis).

4.1.2 Summary of considerations

1. Users should maintain consistency in the environmental conditions of samples and in their handling time. Ideally, exit procedures and subsequent sample collection steps should be performed in a tightly controlled environment.
2. An appropriate assessment of cell condition and cytotoxicity should be included to anchor the molecular measurements to an observable phenotype and ensure that the omics analyses are only conducted on an appropriate percentage of viable cells.
3. Incorporating appropriate control samples such as process (extraction) blanks / no-template controls in the toxicological experiment is advised.

4.1.3 Reporting

Study exit also involves accurately describing the status of the biological test system at the end of the exposure period and time when sampling begins. This includes reporting descriptors of the cells and their conditions to ensure that the assays and results are reproducible. The recommended key descriptors of study exit to be captured and reported for *in vitro* test systems from the OORF (OECD, 2023a) are listed in Table 4 of Section 7 of this document.

- Describe the type of biological sample that will be used for omics analyses (Table 4; reporting field 2.6.1).
- Record and report characteristics of the *in vitro* test system at the time of sampling, e.g., cell density, growth phase and culture passage (Table 4; 2.6.3).

4.2 Sample collection

The sample collection procedure for *in vitro* studies typically requires (1) isolating the cells from the culture media with (2) the option of retaining the culture media for further analysis.

For the omics analysis of cell-based *in vitro* assays, particular attention should be given to obtaining sufficient cell quantity for the intended downstream analytical platform (Section 3.3). Users should consider the sample quantity requirements for the analytical processes and adjust the sampling protocols accordingly (e.g., by pooling replicates, or using larger culture vessels). Ideally, the yields should exceed the amounts that are required for the omics analysis.

For transcriptomics, typical requirements for RNA sequencing are in the region of >100 ng of high-quality total RNA in >10 µL of RNase-free storage solution. Readily available RNA extraction kits typically require <500,000 cells, but >0.5 – 1 million cells may be needed for low yield cells like differentiated primary cells or if the intent is to look for rare transcript. Please note that the minimum amount of sample (i.e., total RNA) and its quality for subsequent sequencing is application- and platform-dependent. Therefore, it is strongly recommended that a library preparation kit provider be consulted for recommendations. Other low-input preparation options are also available (Schuierer *et al.*, 2017), including non-traditional downstream approaches to RNA isolation (e.g., direct lysis) and sequencing (e.g., TempO-Seq, 3-prime RNA-Seq).

For proteomics, the requirements differ by method, particularly when sampling cells and media from *in vitro* test systems (Feist and Hummon, 2015). In label-free quantification for shotgun (bottom-up) global proteomics, the recommended starting amount is approximately 25-100 µg of total protein, which can be obtained from approximately 1-10 million cells or from the culture media in *in vitro* studies. For label-based quantification methods, such as isobaric tagging (e.g., TMT, iTRAQ), 10-100 µg of total protein per sample is required for optimal quantification, depending on the manufacturers labelling instructions, complexity of the sample, and the sensitivity of the mass spectrometer. For less complex samples - such as immunoprecipitated samples, media, and subcellular fractions - a lower protein amount may be sufficient, as they contain fewer proteins and a narrower dynamic range. For targeted proteomics techniques like selected reaction monitoring (SRM) or parallel reaction monitoring (PRM), each measurement typically requires a specific amount of total protein, which varies based on the target protein's abundance, the chosen methodology, and the number of targets. Analyses targeting post-translational modifications (PTMs), such as phosphorylation or glycosylation, often require considerably larger amounts of starting material due to the need for enrichment steps. While 20–100 µg of total protein may suffice in some cases, PTM-focused studies can demand 100–500 µg or more, depending on the specific PTM, the enrichment efficiency, the desired depth of coverage and the applied SOP. Importantly, the recommended starting material for proteomic analyses is also strongly influenced by the choice of protein digestion strategy such as in-gel digestion, filter-aided sample preparation (FASP), or S-Trap-based methods as well as the sensitivity and resolution of the mass spectrometry platform employed. Recent advancements in mass

spectrometry technologies, including systems that integrate ion mobility separation, enhanced nano-electrospray ionisation, advanced quadrupole time-of-flight (Q-TOF) architectures, asymmetric trackless analysers, and segmented linear ion trap designs, have substantially improved detection limits and overall data quality. These innovations facilitate deeper proteome coverage and, in many cases, reduced sample input requirements.

For mass spectrometry metabolomics, requirements depend strongly on the chosen methodology with direct infusion mass spectrometry offering higher sensitivity and requiring approximately 20,000 to 100,000 cells per sample (Malinowska *et al.*, 2022). For more widely implemented LC-MS metabolomics studies, between a few hundred thousand to a million cells per sample are typically sampled (Annex 2, SOPs 4-5). Nuclear magnetic resonance spectroscopy-based approaches typically use a few million cells per sample.

Cell media may contain test substance(s) and many extracellular biomolecules that can significantly adulterate the biomolecular profiles (Section 2.2.1). During sample collection, it is essential to remove this media and to isolate the cells for later processing steps, which ensures that only intracellular biomolecule levels are characterised. There is also an option of retaining the cell media for alternative analysis, including for toxicokinetics (e.g., determining the clearance rate of test substances) as well as omics measurements to investigate the cellular responses to exposure. The latter is possible as the "spent" cell media (also referred to as the secretome or conditioned media) may contain secreted factors and metabolites released by the cells. The omics measurements of spent cell media can provide insights into the cellular responses that are complementary to direct omics measurements of the intracellular biomolecules. For the case of measuring metabolites, the former approach may be referred to as 'metabolic footprinting' and the latter method as 'metabolic fingerprinting' (Araujo *et al.*, 2021). When sampling spent cell media for subsequent metabolomics analysis, one should ensure that the cells are not disturbed during cell media removal. The volume to be collected will depend on the format of the cell culture (e.g., 96- or 384-well plate formats). However, users should prioritise the completion of cell sampling to maintain sample integrity before proceeding with media collection.

During the collection of *in vitro* samples for omics assays, care should be taken to process the culture vessels in a consistent manner. Additionally, multi-well plates should be handled under controlled environmental conditions to prevent further sources of environmental variation (e.g., due to temperature) (Section 2.2.3). Adherence to sterile conditions is strongly advised when possible. Handling the plates one at a time avoids delays that may otherwise negatively impact sample integrity and alter biomolecule abundance. Moreover, handling plates individually minimises batch effects created by processing groups of plates (Section 2.3). Sampling times should be recorded to indicate if any culture vessels have significantly deviated from the collection times of other plates, which may produce unwanted technical variations in the data among the samples (Section 2.2).

Care should be taken during the sampling phase to ensure that all sample collection vessels are appropriately labelled with unique identifiers using ink and/or labels that are compatible with downstream processes (e.g., suitable to withstand liquid nitrogen, if used later in the pre-analytical processing). Additionally, the type of sample container should also be appropriate for downstream processes and the omics application, e.g., the use of DNase-/RNase-free plasticware is recommended for transcriptomics (Section 2.2.4).

4.2.1 Recommended procedures

1. Remove the cell media from each culture vessel (e.g., well of the multi-well plates) and transfer media (without the cells) to the appropriate vessel(s) for the optional downstream analysis of the media (Annex 2, SOP 4.13, 5.06); otherwise, discard the media (Annex 2, SOP 7.01).
2. Proceed directly to sample processing (Section 4.3). If retaining the cell media for further analysis, first proceed with the collection and processing of cellular material and then return to the media with the following steps:

a. Collect an appropriate volume of the supernatant specific to the downstream protocols (Annex 2, SOP 4.13, 5.06).

b. [Optional] Centrifuge the supernatant to remove residual cells and debris (Annex 2, SOP 4.13).

c. Transfer the sample media (minus the pelleted debris) to a labelled and compatible sample tube.

d. Preserve the media sample (Annex 2, SOP 4.13, 5.06).

4.2.2 Summary of considerations

1. Users should ensure that sufficient material is sampled for omics analysis (ideally more than the amount that the analytical steps require).
2. To reduce possible sources of chemical and biological contaminants, remove the media to isolate the sample of cells. [Optional] Media may be retained for further analysis, but the collection of the cells takes priority over the media to maintain sample integrity.
3. A rapid and consistent timeline should be implemented for the collection of all samples.
4. Same environmental conditions should be maintained throughout the sampling procedure with particular attention to temperature. Consistency in culturing and handling biological replicates (e.g., across several weeks of experiments) should also be maintained.
5. Appropriate grade reagents and labware should be used throughout this process. If uncertain, contact the analytics provider for advice.
6. Care should be taken to maintain consistent labelling of samples and adherence to SOPs.

4.2.3 Reporting

The sample collection process also includes accurately reporting the applied methods and the environmental variables. The key recommended descriptors to be documented are outlined in the OORF (OECD, 2023a) and listed in Table 4; 2.6.2-2.6.4 and 2.6.7.

- Describe the methods used for collecting the biological sample(s) (Table 4; 2.6.2-2.6.3).
- Report details of the type of sampling vial or tube that is used (ideally from the same batch/lot number) (Table 4; 2.6.4).
- Where applicable, describe the procedures covering the pooling or aliquoting of samples (Table 4; 2.6.7).
- Thoroughly document sample labels, which should be unique for each sample.

4.3 Sample processing

The processing of *in vitro* cell samples for omics analysis typically includes (1) washing the cells to ensure that residual cell media is removed, (2) quenching the samples, and (3) the option of dissociating cells from the surface of the culture vessels for transfer to another vessel.

For *in vitro* assays, it is imperative to remove any remaining extracellular contaminants, which remain a source of unwanted biomolecules after sampling (Section 2.2.4). This can be achieved by washing the cells with a suitable reagent of appropriate analytical grade (e.g., 0.9% NaCl or sterile buffer solution). For adherent cells cultured in multi-well plates, this wash is performed within the multi-well plate, immediately after the removal of the culture media, by the addition of wash solution to each well, which is then discarded. Care should be taken to ensure that wash solutions do not cause cell lysis and hence should be adapted to the same ionic strength of the culture medium used. Care should also be taken during the

processing phase to ensure that all reagents used during the washing steps are at an appropriate temperature and of an appropriate analytical grade to not compromise sample integrity and/or introduce sources of contamination. Ideally, the reagents should be cooled (e.g., 1 hour on wet ice) prior to their use for washing to prevent sample degradation. Multiple washes may be required to ensure the integrity and reliability of the resulting omics data by effectively removing external contaminants, depending on the omics technology (e.g., metabolomics and proteomics) and the cell type (particularly whether the cells are in suspension or adherent). For cells in suspension, first the cells must be centrifuged, then a wash solution added, and the cells centrifuged again to remove the supernatant. The quenching of samples (typically by freezing) is then required to limit ongoing (bio)chemical reactions and maintain sample integrity. The washing and quenching steps may be coupled to allow a more rapid sampling of cells in suspension, i.e., by adding a quenching solution such as 60 % methanol at –40 °C to –50 °C directly to the cells and cell media, and then centrifuging to remove the supernatant (Canelas *et al.*, 2008). When choosing the appropriate sampling procedure, one should consider susceptibility of the specific cell type to lysis to avoid intracellular metabolite leakage to the supernatant.

Next, adherent cells may either proceed through the remaining collection processes within the multi-well plate, or users may need to dissociate cells from the original experimental culture vessel if downstream analytical procedures do not include in-plate extraction methods. Table 3 lists several procedures used to dissociate (or detach) adherent cells from well plates, including scraping, sonication, and enzymatic dissociation. For the dissociation of adherent cells, users should consider the compatibility of dissociation agents on downstream processes and analytical methods. Use of trypsin to dissociate cells for subsequent metabolomics experiments is not advised as it has been shown to cause metabolite leakage (Bi *et al.*, 2013). If samples remain in the original culture vessel, (i.e., a multi-well plate) this should be sealed to minimise evaporation and cross-well contamination, e.g., using a heat sealer with minimum temperature and duration to achieve containment while reducing the risk of temperature-induced stress to the cells. If cells are dissociated from the original multi-well plate, they are transferred to an appropriate labelled sample tube compatible with both the intended storage procedures and omics approaches. Following cell transfer, a centrifugation step may be required to pellet the cells and remove the supernatant, concentrating the sample for downstream processing. Samples could be pooled at this stage, if required to achieve sufficient sample size. Where cells have been dissociated and transferred to tubes, these may be flash-frozen in liquid nitrogen. For transcriptomics analysis, RNA stabilisation reagents (also known as RNA stabilisers) can be used (Section 3.4).

It is vital that the sample processing steps are performed in a systematic manner across all samples to prevent adversely altering the biomolecular profile or interfering with downstream analyses. Users should consider the consistency of wash/filter steps, performing them for the same duration and under the same environmental conditions. Additional procedures, such as sealing the multi-well plates, should also be consistent to reduce sample variation. Attention should be paid to completing the sample processing steps in a limited amount of time to prevent unwanted degradation of the samples. The type of sample vessel, including plate seals, should also be appropriate for downstream processes (e.g., cryogenic storage) and the intended omics application.

The processing of cells in suspension will require alternative procedures. In place of potential dissociation procedures, cell suspensions may be centrifuged to retain only the cellular material by discarding the supernatant and then applying wash solution(s). Alternatively, a pre-cooled quenching solution can be added directly to the cells in suspension. This is followed by mixing, centrifugation of the sample, removing the quenching solution and resuspension of the cell pellet in an organic solvent (Sellick *et al.*, 2011).

Table 3. Example of procedures used for dissociating (or detaching) adherent cells from well plates.

Procedure	Dissociation agent	Applications
Shake-off, pipetting, or mechanical agitation	Gentle shaking, vigorous pipetting, or vortexing	Loosely adherent cells
Scraping	Cell scraper	Cell lines sensitive to proteases; note that this may damage some cells
Enzymatic dissociation	Trypsin, dispase	Strongly adherent or high-density cultures; note that trypsin is not recommended to dissociate cells for metabolomics
Sonication	Ultrasonic device	Alternative to enzymatic dissociation for strongly adherent or high-density cultures

Other available sample processing options that are specific to transcriptomics include TempO-Seq, which is based on RNA hybridisation and sequencing of gene-specific detector oligonucleotides (Harrill *et al.*, 2021a). This and other approaches (Ye *et al.*, 2018) require no sample (RNA) extraction during the downstream analytical step (Figure 1). Therefore, it can produce gene expression data from processed samples in multi-well plates that omit the cell dissociation step. Instead, processing involves washing the cells, then adding a cell lysis buffer followed by an incubation step, then freezing for sample storage in a state ready for sequencing.

The recommended procedures and considerations below apply to all three types of omics (transcriptomics, proteomics and metabolomics).

4.3.1 Recommended procedures

1. Following the removal of media as described earlier (Section 4.2), wash the wells with pre-cooled reagents (Annex 2, SOP 7.04):
    a. Multiple washes may be included (e.g., two washes with ice-cold 0.9% NaCl or a buffer such as phosphate buffered saline (PBS) for transcriptomics and proteomics, or 150 mM ammonium acetate for metabolomics (as PBS leads to phosphate-related adducts and ion suppression in subsequent LC-MS metabolomics analysis).
2. [Optional] Dissociate the cells from the surface of the culture vessel as proposed below. Please note the following: other examples of procedures for cell detachment are included in Table 3, and cell collection using trypsinisation is generally not recommended for subsequent transcriptomics or metabolomics analysis (Vrtacnik *et al.*, 2014; Bi *et al.*, 2013).
    a. Add an ultra-cooled quenching/extraction solution, which will depend on the downstream omics analysis (e.g., Annex 2, SOP 4-5) and swirl to distribute within each well.
    b. Float the plate on a dry ice/isopropanol bath for 10 min.
    c. Allow the cellular material to thaw to a slurry and scrape the cells.
    d. Transfer the cell slurry to an appropriately labelled 2 mL microcentrifugetube.
3. If cells are retained in a multi-well plate for in-plate extraction:
    a. Seal the plate (e.g., Annex 2, SOP, 7.05) with compatible foils.
    b. Incubate the plate on dry ice for 15 minutes to quench the samples (Annex 2, SOP 7.06).

4. If cells are dissociated and transferred to new tubes:
    a. Flash-freeze the samples in vials using liquid nitrogen. [Optional, if transcriptomics] Preserve the tissue sample in an RNA stabilisation reagent.

4.3.2 Summary of considerations

1. The cells should be washed and/or filtered to remove all the culture media and any contamination from extracellular biomolecules.
2. [Optional] For the dissociation of adherent cells from the culture vessel and transfer to a new sample tube, the dissociation agents must be compatible with the downstream analytical processes.
3. When the samples are retained within multi-well plates, these should be appropriately sealed to minimise sample loss or contamination with seals compatible with the intended storage temperatures.
4. Quenching of samples should be conducted for most sample types to arrest cellular (e.g., enzymatic) processes using either liquid nitrogen or dry ice.
5. Attention should be paid to limiting the duration of sample processing steps, ensuring consistency, and to generally using lower than ambient temperature to maintain sample integrity and minimise variability.
6. The appropriate grade wash solutions, reagents and labware (e.g., sample tubes or plates) should be used consistently throughout the sample processing steps.

4.3.3 Reporting

Thorough documentation and reporting of sample processing steps are recommended to promote study reproducibility and facilitate the assessment of the reliability of data including capturing possible sources of unwanted variation that may affect sample and/or data quality. Example reporting elements outlined in the OORF (OECD, 2023a) include:

- Document any exogenous chemicals or reagents (if present) within the samples (Table 4; 2.6.4).
- Describe any washing procedures applied, including the timing of steps and use of solvents (Table 4; 2.6.5).
- Report the quenching procedure, including the timing of steps and the reagents used (Table 4; 2.6.6).
- Where applicable, describe the procedures covering the pooling or aliquoting of samples (Table 4; 2.6.7).

4.4 Sample storage and transportation

The omics analytical steps (Figure 1) are typically performed within facilities that are at different locations to where the pre-analytical steps are carried out and sometimes at much later dates than the sampling. The following considerations for the storage and transportation of samples, introduced in Sections 3.5 and 3.6, respectively, apply to all *in vitro* omics investigations to preserve the integrity of the biomolecules.

4.4.1 Summary of considerations relevant to in vitro sample storage

1. The long-term integrity of the biomolecules can be maintained by storing the samples at –80 °C (or lower). Users should ensure that they have access to appropriate facilities to achieve this.

2. Careful consideration should be given to sample labelling, tracking, and documenting information about the storage conditions throughout the life of a sample.
3. Freeze-thaw cycles should ideally be avoided, but, if necessary, they should be kept at a minimum, consistent across all samples and well-documented. Users may consider aliquoting the samples prior to storage to avoid freeze-thaw cycles.

4.4.2 Summary of considerations relevant to in vitro sample transport

1. Particular attention should be given to ensuring that sample temperature is appropriate and consistent throughout shipping to preserve sample integrity. For example, sealed plates or sample tubes should be shipped on dry ice.
    - Ensure that there is a sufficient volume of refrigerant to hold a consistent temperature for at least three days when shipping can be achieved within one day.
2. Select the appropriate packaging that maintains environmental conditions and prevents damage to the samples.
    - Sample vessels should be double-contained in sealed bags rated for low temperatures.
3. Users should pay close attention to correctly labelling packages and completing all necessary paperwork (e.g., customs-related) to ensure minimal shipping delays.
    - Plan the shipment to avoid delivery near weekends and holidays.
    - Retain all documents indicating date, time, and signature(s) in the study files.
    - Notify the receiver at least 24 hours in advance of the planned shipment with the package tracking number; send the receiver an electronic version of the inventory list.
4. Upon receiving the package, it should be carefully examined, and any issues should be recorded (e.g., evaporation of dry ice).

4.4.3 Reporting

- It is advisable to report the following conditions when the samples are stored and transported, following the guidance provided in the OORF (OECD, 2023a) and listed in Table 4:
    - Document the temperature and duration of sample storage (Table 4; 2.6.8).
    - Provide a description of the method of transport (Table 4; 2.6.8).
    - Where relevant, record the number of freeze-thaw cycles that samples are exposed to (Table 4; 2.6.8).
    - Record any instances of thawing during transport (Table 4; 2.6.8).

5 Guidance on sampling blood and tissue from in vivo test systems

The biological materials of an *in vivo* toxicity test are obtained from experiments conducted on whole organisms by surgically dissecting the target tissues or by sampling blood, thereby providing sufficient mass or volume to subsequently extract a desired amount of RNA, protein and/or metabolites for omics investigations. This section of the Guidance Document provides advice on how to obtain samples that generate high-quality omics data. The circumstances where animal testing is conducted to fulfil existing regulatory requirements create an opportunity to preserve samples and generate omics signatures without additional animal testing.

There are four broad types of animal tissues for sampling: connective tissue, epithelial tissue, muscle tissue, and nervous system tissue. Their structure and composition can present challenges when isolating biomolecules during the analytical stages (Figure 1), which can be partially overcome by considerations taken during sample collection, sample processing and sample storage (further examples under Section 5.2).

The sampling of solid tissues from *in vivo* studies requires precision to exclude non-target tissues during dissection, which should be done by experienced staff trained in histological techniques. It is important to consider the challenges of achieving consistent sampling across animals or tissues, particularly given the sensitivity of omics measurements. In case a larger sample is collected and stored at necropsy, it may need to be cut down (while frozen) prior to extraction. Care should be taken to avoid sampling error, and always the same region should be sampled. If this is not possible, the whole preserved tissue piece needs to be pulverised. Pulverisation allows processing of only part of the powder, representing the same mix for all samples to be compared.

Sample collection procedures for omics are necessarily different for blood, which is especially challenging because blood is a complex matrix of cells and biofluid, which needs processing to separate them prior to storage and downstream analysis. For instance, blood can be processed to yield either serum or plasma, which differ in composition and in the methods used to prepare them. Moreover, blood has many other properties that must be considered for omics sampling:

- Blood is rich in enzymes; hence, specialised processing is required to preserve metabolite profiles (Section 2.2.1).
- Red blood cells (which make up the largest fraction of cell types) contain large amounts of haemoglobin, which inhibits reverse transcription for transcriptomics.
- Plasma has a high concentration of RNases that must be quickly inactivated. Freeze-thaw cycles are especially damaging to the plasma membrane, releasing RNases from the ruptured cells into the plasma (Section 2.2.2).
- If blood is to be preserved for transcriptomics, it is recommended that peripheral leukocytes are removed before storage or that the blood is stored with special RNA-stabilising reagents (Section 3.4) to safeguard RNA integrity.

Although such properties create difficulties in ensuring sufficient abundance and/or integrity of the biomolecules obtained from blood during omics sampling, they also offer opportunities when special considerations are taken. For example, the aqueous sample type (after separation from the cells) is rich in peptides, proteins, carbohydrates, lipids, amino acids, electrolytes, and other functional small molecules. Hence, these are suitable for metabolomics and proteomics investigations.

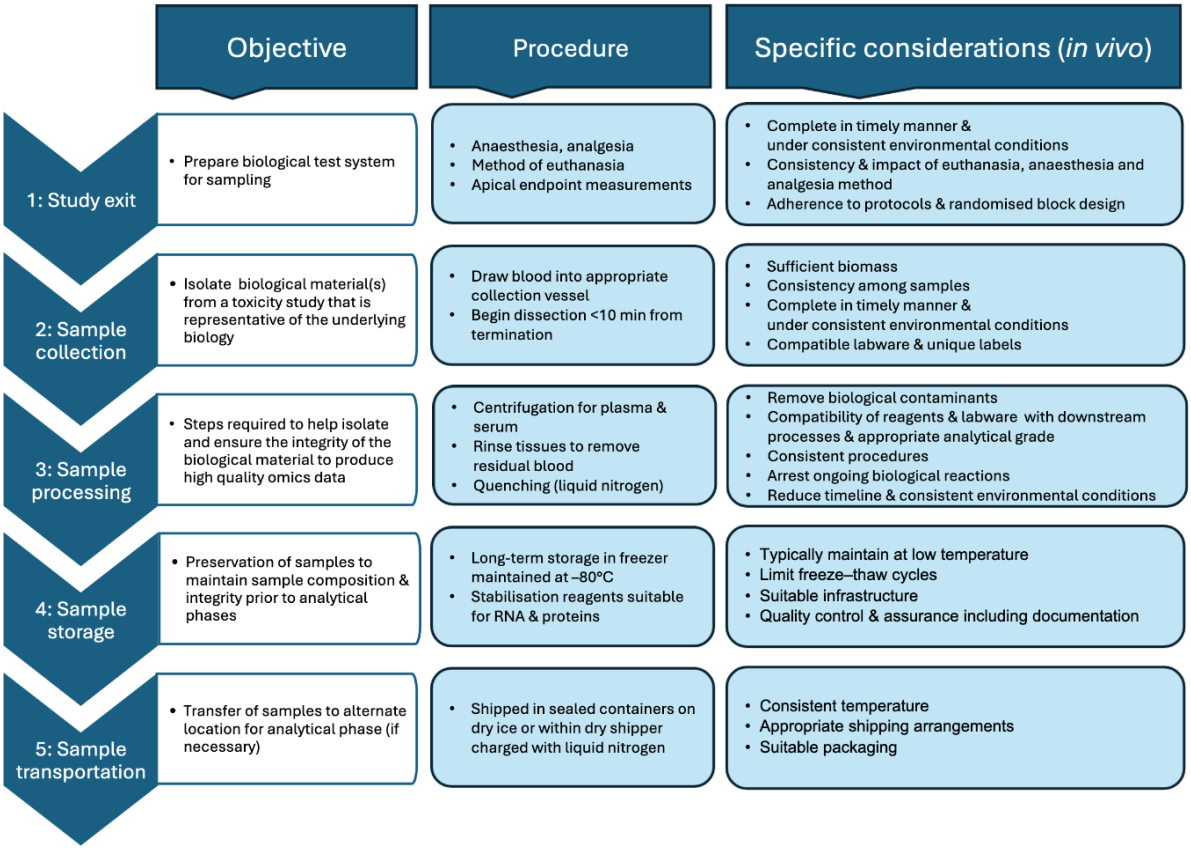

Figure 5. Main objectives, recommended procedures and specific considerations of the five pre-analytical processes used during sampling of blood and tissues from in vivo studies for omics analyses.

Given the biomolecular richness and dynamics, users may consider collecting blood samples during the exposure period, e.g., at specific time intervals (e.g., every 2 hours following the first dose), to provide toxicokinetic estimates (see Section 5.2). This may be performed via micro-sampling (Prior *et al.*, 2025), alleviating the stress and welfare concerns associated with routine blood collection, potentially compromising the reliability and validity of the data (Chapman *et al.*, 2014). Micro-sampling can reduce the experimental burden and allow researchers to consider ethical options in limited research settings. However, the lower volumes collected may not be compatible with all omics applications.

The following subsections detail specific considerations and example procedures for the five pre-analytical processes of sampling for omics that may be applied when collecting terminal tissue and blood from *in vivo* studies for omics analyses (Figure 5). General considerations are first presented that should be considered when designing and conducting any omics study. These are followed by example procedures with optional components, which are intended to help guide users in designing their own procedures, suitable for their own test systems and downstream analytical options. The example procedures in this section are based on omics sampling from chemical toxicity testing using rats.

5.1 Study exit

The study exit procedures when samples are collected from *in vivo* studies typically require (1) terminating the experimental phase and preparing the test organisms for the collection of blood and/or tissue samples, and (2) measuring apical endpoints where appropriate. Study exit procedures will, however, vary depending on whether the terminal sample collection is conducted at the end of the exposure period or whether samples are collected during the on-going exposure period (e.g., by micro-sampling). The following guidance focuses on the process of terminal sampling.

Anaesthesia is administered prior to the collection of blood and tissue samples. All forms of anaesthesia will result in changes in physiology producing detectable changes to the omics profile. Consistency in this process and in the handling of all organisms is essential to prevent the introduction of unwanted (confounding) omics variations. Therefore, users should be aware that applying an equivalent dose of the same analgesic or anaesthetic drugs, even with the necessary strict adherence to protocols may not consistently influence gene expression, protein production, or metabolism in the same manner across individual animals. When inter-study comparisons are intended, the same methods should be implemented. Additional reporting on the state of the organisms including their responses to the analgesic or anaesthetic drugs and on the details of the test procedures are recommended (Section 7, Table 4: 2.6.2) to facilitate reproducibility (e.g., strain, source and age of test organisms), and for compliance with welfare standards, such as housing conditions, feeding regime, and rationale for dose selection. Also, the anaesthesia and euthanasia should be performed in a highly standardised way to reduce variability. Given the variability in how animals may respond to anaesthesia and/or euthanasia, it is important to observe and report if animals show any unexpected responses, as this may influence omics study outcomes by introducing confounding factors.

Users should carefully consider the impact of the time that is taken to perform these steps and how it may affect the downstream omics data quality. For example, when the sampling for omics is paired with other applications (e.g., histopathology), additional time may be required to precisely dissect/divide tissues. The time intervals between organ excision, phenotyping, and the processing of the sample to preserve the experimental biomolecular profile should be minimised and standardised. Long time intervals allow ongoing cellular and biomolecular changes within the sample to continue, including endogenous (bio)chemical reactions, which may result in a biomolecular omics profile not entirely representative of the experimental treatment effects (Section 2.2.1). Furthermore, long time intervals may elevate the risk that the samples will also undergo exposure to alternate environmental conditions (e.g., hypoxia after tissue excision). Additionally, when combining other applications and omics studies, it is essential to consider the potential limitations of e.g., tissue availability (Annex 3). The priority of various analyses such as apical endpoint measurements and omics should be considered for the study design to not compromise the integrity of planned analyses.

To maintain the timing and conditions consistent among the test subjects, the study exit, and sample collection may need to extend over two or more days. When possible, users are advised to divide the tissues that are then handled separately for omics sampling and for any other applications such as phenotyping. A multi-day study exit elevates the risk that procedures are handled by more than one person, which should be avoided if possible. Otherwise, care should be taken to ensure that all persons involved have the same appropriate level of training and follow the same SOPs. A randomised block design is advised to minimise the unwanted potential sources of systemic bias within a multi-day study exit (Section 2.3). Moreover, an envisioned multi-day study exit has direct implications on the study design and needs to be addressed accordingly (e.g., by shifted dates for the study start or by accounting for circadian rhythms in biological responses).

Many of the *in vivo* studies, from which samples for omics measurements could be obtained, will be studies that are performed to fulfil a regulatory requirement. These studies are usually conducted in accordance with guidelines (such as outlined in OECD Test Guidelines) under Good Laboratory Practice (GLP). If

omics sampling is conducted in such an *in vivo* regulatory guideline study, the measurement of apical endpoints such as organ weights, as well as preservation of organs and tissues for histopathological evaluation is mandatory to ensure that the regulatory validity of the study is not compromised and has priority over sampling for omics measurements (see also Annex 3).

The recommended procedures and considerations below apply to all three types of omics (transcriptomics, proteomics and metabolomics).

5.1.1 Recommended procedures

1. Record the fasting time (Annex 2, SOP 2.01). If applicable, administer anaesthesia and/or perform euthanasia using methods and protocols that are consistent for inter-study comparisons (Annex 2, SOP 1.03).
2. Record the light/dark cycle and ensure an acclimatisation period.
3. [Tissue only] Note the time of euthanasia and organ excision.
4. Collect apical endpoint measurements.

5.1.2 Summary of considerations

1. Users should carefully consider the impact of any anaesthesia and analgesia administered on the omics data, focussing on the consistency and compatibility of procedures with downstream applications.
2. A consistent method of euthanasia should be applied both within and among studies. Describe the biological test system at the time of sample collection.
3. Users should follow protocols with precision, performing tasks consistently to avoid systemic bias (batch effects) and/or implement an appropriate randomised block design for large studies.
4. Users should maintain consistency in the environmental conditions and time taken when handling organisms and samples. Ideally, exit procedures and subsequent sample collection steps should be performed in a tightly controlled environment.
5. The collection of apical endpoints, when appropriate, may be included to anchor molecular measurements to an observable phenotype.

5.1.3 Reporting

Study exit involves accurately describing the status of the biological test system at the end of the exposure period, the condition of the samples, and the time when organs/tissues are extracted. This includes reporting key descriptors of the phenotypic observations and apical endpoints in either a GLP-study report or captured and reported for *in vivo* test systems using the OORF (OECD, 2023a) as described in Table 4.

- Describe the type of biological sample that will be used for omics analyses (Table 4; 2.6.1). Record the method(s) of anaesthesia, analgesia and/or euthanasia performed, including the substance, dose, route of administration, and timing of steps (Table 4; 2.6.2).
- Observe and report if animals show any unexpected responses to anaesthesia, analgesia and/or euthanasia that could significantly influence omics study outcomes by introducing confounding factors (Table 4: 2.6.2).
- Record the phenotypic measurements and apical endpoints, including the time when these observations were made in a timely and consistent manner (Table 4; 2.6.2).

5.2 Sample collection

The sample collection procedure for *in vivo* studies typically requires (1) dissection of the target organ/tissues, and (2) precise isolation of the biological sample used for omics analysis (i.e., blood and/or solid tissue).

Sampling blood

When conducting omics analyses on blood samples from *in vivo* studies, particular care should be taken to prevent haemolysis (the destruction of red blood cells), as it is one of the most common pre-analytical errors in omics research (Safari *et al.*, 2023). It results in the release of proteins (e.g., haemoglobin) and metabolites into the serum or plasma. It may, therefore, drastically alter profiles obtained from metabolomics and can also affect gene expression profiles (Michalska-Falkowska *et al.*, 2023). It may also affect small RNA profiles, particularly microRNAs (miRNAs), by introducing RNA species from lysed red blood cells, potentially confounding transcriptomic analyses (Kirschner *et al.*, 2011). A visual assessment of haemolysis should be performed during blood collection and may follow a three-point scale (absent, suspected, present). Additional spectrophotometric analysis of oxyhaemoglobin absorbance (λ= 414 and 385 nm) may be helpful to quantify the extent of degradation (Michalska-Falkowska *et al.*, 2023).

The type of collection vessel should be compatible with downstream omics analyses. For example, blood sample tubes containing clot activators and anticoagulant additives may have different shortcomings or efficacy depending on the application and, therefore, may not be suitable for all applications (Rai *et al.*, 2005). To reduce variability and to achieve standardisation, it is recommended to always use the same type of sampling tubes within one experimental set-up or research question. Tubes designed for sampling human newborn babies or infants may be appropriately used (Rai *et al.*, 2005).

For metabolomics, the volume of blood that is required depends on the subsequent procedure for separating serum/plasma and on the scope of the metabolome measurements. To prepare plasma, for example, only less than half of the sampled blood volume consists of plasma. Therefore, 120-150 µL of blood must be sampled to obtain 60 µL for plasma metabolite extraction. If additional analytical platforms are employed (e.g., to measure specific groups of metabolites, such as hormones, bile acids, etc.), additional volume is needed. Typically, proteomics requires a higher blood volume compared to metabolomics, i.e., for serum/plasma 100 µL are recommended, equating to about 200-250 µL of blood. Depending on the type of omics employed, serum and/or plasma sample requirements can range from as low as 20 µL to 450 µL per platform (e.g., Annex 2, SOP 3.01 and 9.01). Blood can be aliquoted as part of the sample extraction procedure, and therefore, sampling precise volumes may not be required (see also sample processing).

Users may consider collecting blood samples during the exposure period at specific time intervals (e.g., 2 hours following the first dose) to provide toxicokinetic estimates. This may be performed via micro-sampling to reduce total burden and improve animal welfare. However, the lower blood volumes (≤ 50 µL) may not be compatible with all omics applications. Also, the sampling of larger blood volumes (≥200 µL) could cause anaemia or other secondary effects such as bone marrow and haematological changes, which may confound interpretation of effects (Chapman *et al.*, 2014) and potentially compromise the study.

Sampling solid tissue

During tissue sampling for omics, the integrity of the cells and of the biomolecules begin to be compromised when the vascular supply to an organ is interrupted during surgery (warm ischemia) or when tissue is removed and placed in a cold container (cold ischemia) (Snapes *et al.*, 2023). Ischemia time (whether warm and/or cold) can induce molecular effects due to hypoxia and stress (Section 2.3). Users are advised to record details (or deviations from the SOPs), such as the time of resection start, vessel ligation, resection

end and tissue sample preservation, which are essential for monitoring warm and cold ischemia, and where possible, minimise the time taken for sample collection (Michalska-Falkowska *et al.*, 2023). Ideally, sample collection should begin within 10 minutes of the termination of the study (Annex 2, SOP 1.07). Dissection of fresh tissue samples should be performed on ice.

During dissection, the tissues should be taken from the same anatomical site of each test subject; this is particularly relevant when obtaining organs with complex substructures (e.g., the kidney). When tumour tissues are collected, users should consider mosaicism, where specimens may also include normal tissue. In such instances, additional pathological evaluation prior to analysis of samples may be helpful. In addition, the structure and composition of certain tissues can present challenges when isolating biomolecules. This can be partially overcome by refining strategies based on the structure and composition of the tissue during sample collection, sample processing and sample storage. For example, when sampling materials for transcriptomics, the following factors should be considered:

- Biomolecules are most difficult to extract from muscle tissues such as the mammalian heart. As muscle tissues are polynucleated and have low cell density, the total RNA yield is typically low, thereby requiring more mass than other tissues. Fibrous tissues are difficult to homogenise completely, which can result in degraded RNA and even lower yield. However, higher yield and RNA integrity can be achieved by adjusting the sample mass and by pulverising the tissue into a powder while the tissue is kept frozen. Therefore, storing the sample at low temperature (–80 °C or lower) is recommended.
- Brain tissues are rich in lipids, which can complicate the RNA extraction process. Other organs such as the spleen and thymus are high in nucleases and nucleic acids. Degradation can be prevented by pulverising the tissue into a powder while the tissue is kept frozen, inactivating the nucleases found in the tissues.

Distributing sample material to enable omics analysis and histopathology or for multiple omics analysis can be straightforward for blood by following a split sample design. However, this design becomes more complex for solid tissue due to regional differences in tissue composition. To avoid bias, several strategies are proposed (Canzler *et al.*, 2020): (1) tissue can be homogenised and evenly distributed across omics technologies, (2) sections from a cryo-microtome can be consistently assigned to different omics analyses, or (3) organ or tissue segments can be systematically allocated for each omics analysis. Such strategies can be applied for studies from which samples should be generated for different omics analysis. However, these strategies may not be suitable for the regulatory TG studies, where standard parameters such as histological samples have priority over omics samples (see also Annex 3).

Care during the sampling of solid tissue should be taken to avoid sources of biological contamination (Section 2.2.4), including clean techniques and RNase-free equipment (Annex 2, SOP 1 and 2). For tissue sampling, each specimen should be handled with clean instruments to avoid contamination, especially when sample yields are low. Particular attention should be given to obtaining sufficient yield from the organ/tissues of the *in vivo* test systems for omics analysis. Where possible, users should collect material in excess to ensure that, if there are technical analytical issues, there is sufficient sample remaining for a repeat of the analysis. This is primarily due to the feasibility and ethical considerations of repeating a study if all the sample is lost. Blood volumes are easier to adjust post-sampling to achieve sufficient sample quantity for analysis. However, tissue sampling requires precise dissection of a limited amount of tissue, depending on the target organ. Users should establish the required sample mass before processing. Furthermore, (Canzler *et al.*, 2020) argue for in vivo studies that since different sampling time points per omics layer would (1) result in non-paired samples, associated with the difficulties in data analysis, and (2) multiply the number of animals required by the number of omics layers, as only one time point can be obtained from one individual, the samples for the different omics layers should be generated at identical time points.

For transcriptomics, typical requirements for RNA sequencing are in the region of >100 ng of high-quality total RNA in suspension within >10 µL of RNase-free storage solution. Other low input and/or low tissue quality preparation options are also available including for samples that are fixed in formalin-based solutions (usually from workflows of standard histopathology studies). In such instances, these preparation options are often accompanied by non-traditional downstream approaches (e.g., TempO-Seq, 3-prime RNA-Seq).

For proteomics, the sample requirements are dependent on the method. In label-free quantification for shotgun (bottom-up) global proteomics, the recommended starting amount is typically 25-100 µg of total protein. For label-based quantification methods, such as isobaric tagging (e.g., TMT, iTRAQ), up to 100 µg of total protein per sample may be required. In cases involving fewer complex samples or highly sensitive mass spectrometers, lower amounts may be sufficient. For targeted proteomics techniques like selected reaction monitoring (SRM) or parallel reaction monitoring (PRM), each measurement generally necessitates a specific amount of total protein, which varies based on the target protein's abundance, the chosen methodology, and the number of targets. Analyses targeting post-translational modifications (PTMs), such as phosphorylation or glycosylation, often require considerably larger amounts of starting material due to the need for enrichment steps. While 20–100 µg of total protein may suffice in some cases, PTM-focused studies can demand 100–500 µg or more, depending on the specific PTM, the enrichment efficiency, the desired depth of coverage and the applied SOP.

Importantly, the recommended starting material for proteomic analyses is also strongly influenced by the choice of protein digestion strategy such as in-gel digestion, filter-aided sample preparation (FASP), or S-Trap-based methods as well as the sensitivity and resolution of the mass spectrometry platform employed. Recent advancements in mass spectrometry technologies, including systems that integrate ion mobility separation, enhanced nano-electrospray ionization, advanced quadrupole time-of-flight (Q-TOF) architectures, asymmetric trackless analysers, and segmented linear ion trap designs, have substantially improved detection limits and overall data quality. These innovations facilitate deeper proteome coverage and, in many cases, reduced sample input requirements.

For mass spectrometry metabolomics, requirements can range from 50-150 mg of tissue per sample (Annexe 2, SOP 2), although depending upon the analytical platform used samples as low as 1-10 mg can be measured.

A rapid timeline for this process should be implemented to reduce unwanted molecular changes due to environmental conditions (Section 2.2.3) and/or endogenous (bio)chemical reactions (Section 2.2.1). The timing and protocol followed for all sample collection procedures should be consistent within a study to reduce variation among samples (Section 2.3).

The recommended procedures and considerations below apply to all three types of omics (transcriptomics, proteomics and metabolomics).

5.2.1 Recommended procedures (sampling blood)

1. Draw blood under anaesthesia via venipuncture, sublingual vein, retro-bulbar (Annex 2, SOP 3.05), heart or tail vein puncture.
2. The collection vessel will depend on whether plasma and/or serum of whole blood is required:
   - Plasma: see Annex 2, SOP 3 and SOP 9, noting that haemolysis should be avoided.
   - Serum: sampling directly into a vacuette. Process immediately (Section 5.3)

5.2.2 Recommended procedures (sampling solid tissue)

1. Begin dissection with clean instruments, ideally within 10 minutes of terminating the study. Record the start and end times of the dissection (Annex 2, SOP 1.07).

2. Collect a sufficient tissue amount to extract the required amounts of RNA, proteins and/or metabolites (Annex 2, SOP 1.08, 2.02, 8.01, 10.01, 11.01-11.04).
3. Transfer tissue to the collection tube using clean forceps.

5.2.3 Summary of considerations

1. Users should ensure that sufficient sample material for omics analysis is obtained, including being consistent in the anatomical site for the dissection (tissue sampling) and from where blood is sampled for consistent volume (Annex 2, SOP 2.02 and 3.02, 8.01, 10.01, 11.01-11.04).
2. Maintain consistent environmental conditions throughout the sampling procedures including temperature.
3. Collect samples rapidly and consistently following a timeline that avoids sample degradation through long ischemia times (solid tissue) and haemolysis (blood).
4. Consistently use the appropriate grade of reagents and consumables including blood sample tubes (some containing additional reagents) compatible with downstream analysis; if uncertain, contact the analytics provider for advice (Annex 2, SOP 8.02 and 9.04).
5. Strictly adhere to protocols throughout the process, including random sampling.
6. Avoid biological contamination where possible (e.g., handling tissues with forceps).
7. Care should be taken to maintain consistent labelling and adherence to SOPs (Annex 2, SOP 8.03, 9.07).

5.2.4 Reporting

Sample collection also includes reporting the procedures that are used and documenting key recommended descriptors that are outlined in the OORF (OECD, 2023a) and are listed in Table 4; 2.6.2-2.6.4.

- Describe the methods that were used for the collection of biological sample(s) (Table 4; 2.6.2-2.6.3).
- Provide details of the type of sampling vials or tubes to be used (ideally from the same batch/lot number) (Table 4; 2.6.4).
- Thoroughly document the sample labels, which should be unique for each sample.

5.3 Sample processing

The processing of blood samples from *in vivo* studies varies depending on the fraction to be collected. Whole blood may be directly frozen, plasma and serum require additional steps including (1) clotting (serum only), (2) centrifugation to separate the desired fraction, and (3) quenching. The processing of tissue samples typically includes washing to remove residual biological contaminants and quenching of the sample to halt enzymatic processes and significantly reduce confounding changes to the biomolecular profiles.

For *in vivo* assays, sample processing steps should be performed in a systematic manner across all samples to prevent adversely altering the omics profile or interfering with downstream analyses (Gonzalez-Dominguez *et al.*, 2020). Consistency in all processing steps, including their durations between terminating the study to sample storage and the environmental variables including temperature, is essential to avoid inter-sample variation due to differences in sample integrity and composition (Gonzalez-Dominguez *et al.*, 2020).

Processing blood

The processing of blood samples from *in vivo* studies should begin within 30 minutes from the time the sample was collected (at room temperature) and ideally within 40 minutes from the termination of the study (Snapes *et al.*, 2023). It is vital that sample processing steps are performed systematically across all samples to prevent adversely altering the biomolecular profile or interfering with downstream analyses (Gonzalez-Dominguez *et al.*, 2020). The collection tube type may depend on the downstream analyses performed and commercially available options may already include the required additives. Users should take care to follow the manufacturers' instructions when preparing samples, as the inclusion of mixing or tube inversion steps may be required to ensure a homogenous distribution of tube additives through the sample, which, if not achieved, may alter the omics profiles (ISO, 2021). Anticoagulants (e.g., heparin, citrate, EDTA) have been shown to affect metabolic profiles. No single anticoagulant is optimal for metabolomics with strengths and weaknesses for each, e.g., ion suppression/enhancement has been observed for citrate and EDTA; (Gonzalez-Dominguez *et al.*, 2020). The type and amount of anticoagulant must be documented for all studies and consistently applied throughout.

Additional post-collection steps such as centrifugation and filtration are typically required for blood matrices. For serum collection, clotting is required, the duration and temperature of this step may influence the final biomolecular profile and should be consistent throughout the study (ISO, 2021). Centrifugal force can lead to differences in metabolite profiles and should be consistent throughout studies and clearly outlined in SOPs along with the temperature during centrifugation (Safari *et al.*, 2023). It is crucial to assess and report haemolysis in both serum and plasma samples after their separation from whole blood. This can be done, e.g., by visual inspection for a pink or red tinge in the serum or plasma, or quantitative measures of free haemoglobin.

Aliquoting serum/plasma into consistent smaller amounts at early sample collection stage is ideal to avoid freeze-thawing of samples. Furthermore, to allow re-processing and re-measurement in case of technical issues, the sampling and storage of additional back-up material should be considered.

Consistency in all processing steps is required to avoid inter-sample variation caused by differences in the overall integrity of the samples affecting the composition and abundance of biomolecules (Gonzalez-Dominguez *et al.*, 2020). Quenching of the sample is then required to limit ongoing (bio)chemical reactions and maintain sample integrity by flash-freezing in liquid nitrogen. If liquid nitrogen is not available, dry ice may be used instead (although liquid nitrogen is preferred).

Processing solid tissue

The processing of solid tissue samples from *in vivo* studies may involve rinsing the excised tissues in cold saline wash solution (0.9% NaCl) to remove residual biological contaminants (e.g., from blood) that may alter the omics profile (Section 2.3). The type of wash solution, the volume(s) and the washing procedure should be consistent across all samples to reduce technical variations. It is imperative that all liquid is then removed from the sample collection vessel. The inclusion of a thorough blood removal process prior to tissue processing may also be considered for accurate omics measurements. Perfusion is widely considered the most effective technique for minimizing blood contamination in tissues. However, the perfusion itself has an effect on the physiology of the tissue used for omics analysis. Furthermore, in studies where multiple parameters, in addition to omics, are evaluated, perfusion may cause additional confounding factors for other study readouts. The fact that remaining blood in the tissues might have an influence on the data, can be taken into account in the interpretation of metabolomics data. Therefore, the priority of various analyses should be considered for the study design to not compromise the integrity of planned analyses.

If the preservation of the samples is by cryopreservation, the quenching of the samples for all downstream omics applications is achieved by rapidly placing the excised tissue within its storage vessel into liquid

nitrogen at a temperature of –196 °C to halt enzymatic processes and to significantly reduce changes to the biomolecular profiles. Quenching at higher temperatures (e.g., by dry ice) may be acceptable but may not be rapid enough for certain types of dense animal tissues (Section 3.4). Once the samples are frozen, they should not be thawed until the analytical steps are undertaken to extract the biomolecules (Figure 1).

For transcriptomics analysis, RNA stabilisation reagents (also known as RNA stabilisers) can be used instead of quenching (Section 3.4). These commercially available reagents prevent RNA degradation from occurring and they preserve the RNA integrity for longer periods of time, especially when using warmer temperatures for cryopreservation. However, for solid tissues, ensuring full penetration of the stabiliser is critical, as insufficient diffusion may leave inner tissue regions unprotected, leading to RNA degradation during thawing. Homogenisation or mechanical disruption may be necessary for larger tissue samples. Users are advised to follow the manufactures instructions for optimal results.

If the preservation of tissue samples is done via fixation, paired samples may be divided for flash-freezing and fixation. Both methods achieve quenching. However, the type of fixation should be selected to ensure compatibility with downstream analyses (EMA, 2017). For example, formalin-fixed, paraffin-embedded samples generally produce lower quality RNA due to higher fragmentation, biomolecular crosslinks, and occurrence of base modifications (Babel *et al.*, 2020). Fixation may be affected by humidity, oxygenation and temperature in addition to the tissue type and volume (EMA, 2017). Consistency in fixation protocol including the duration should be considered throughout sample processing (Section 3.4).

For multi-omics experiments, paired samples can be obtained from the same tissue by cryo-conservation followed by sectioning into slides. This approach allows for parallel transcriptomics, proteomics and metabolomics analyses while preserving spatial context (Canzler *et al.*, 2020). However, inconsistencies in tissue structure can lead to variability in omics data, emphasising the need for standardised slide preparation. Key parameters such as the sectioning procedure, slide thickness, distribution strategy, and analytical method allocation should be carefully documented to ensure reproducibility and comparability across omics technologies. Additionally, when allocating slides for different omics analyses, care should be taken to minimise batch effects due to sample processing order and potential degradation over time.

5.3.1 Recommended procedures (blood sampling)

Blood

1. Flash-freeze whole blood sample in liquid nitrogen after collection. Alternatively, dry ice may be used instead (although liquid nitrogen is preferred).

Plasma

1. Centrifuge the sample (Annex 2, SOP 3.09, 9.03).
2. Collect the supernatant in a labelled sample collection tube (e.g., Eppendorf tube) (Annex 2, SOP 3.11, 9.04).
3. [Optional] Fill the remaining headspace within the sample tube with inert gas (e.g., Argon) (Annex 2, SOP 3.11).
4. Flash-freeze the labelled sample tube in liquid nitrogen.

Serum

1. Allow the serum sample to clot.
2. Centrifuge sample.
3. Collect all supernatant and transfer to a clean, labelled tube.
4. [Optional] Fill the remaining headspace within the sample tube with inert gas (e.g., Argon).

5. Flash-freeze the labelled sample tube in liquid nitrogen.

5.3.2 Recommended procedures (sampling solid tissue)

1. Rinse the tissue to remove residual blood (0.9% NaCl) prior to placing it in a labelled collection tube (Annex 2, SOP 2.04).
2. [Optional, if transcriptomics] Preserve the tissue sample in an RNA stabilisation reagent (Annex 2, SOP 1.10).
3. Flash-freeze the sample in liquid nitrogen as rapidly as possible after tissue dissection (Annex 2, SOP 1.11, 8.02, 10.02, 11.06).
4. Avoid preserving tissue samples via fixation whenever possible. Instead, paired samples may be divided for flash-freezing and fixation.

5.3.3 Summary of considerations

1. Users should consider washing tissue samples to remove residual biological contaminants.
2. Any centrifugation should be performed in a consistent manner.
3. Evaluate and report any haemolysis in serum and plasma samples after their separation from whole blood.
4. The addition of additives and/or preservatives to the samples should consider their compatibility with downstream analyses and be consistent throughout the process.
5. Quenching of samples should be included in most sample types to arrest cellular processes using either liquid nitrogen or dry ice.
6. Care should be taken to limit the duration of processing steps, occurring often at lower than ambient temperature, and ensure consistency throughout any sample processing to maintain sample integrity.

5.3.4 Reporting

Thorough documentation and reporting of sample processing steps are recommended to promote study reproducibility and facilitate assessment of the reliability of data, including capturing possible sources of unwanted variation that may affect sample and/or data quality. Reporting elements are outlined in the OORF guidance (OECD, 2023a) and are listed in Table 4.

Document any exogenous chemicals or reagents present within the samples (e.g., preservatives added to the sample or chemicals already present within the collection tube, such as anticoagulants) (Table 4; 2.6.4).

- Describe any washing procedures that were applied, including the solvent that was used (Table 4; 2.6.5).
- Report the method used for quenching, including reagents when applicable (Table 4; 2.6.6).
- Where applicable, a description of procedures covering the pooling or aliquoting of samples (Table 4; 2.6.7).

5.4 Sample storage and transportation

The omics analytical steps (Figure 1) are often performed within facilities located at different institutions than the facilities where the pre-analytical steps are carried out and often at later dates (up to several years post-collection). Although storage at –80 °C is generally recommended, small cuts of tissue specimens can typically be preserved for transcriptomics using RNA preservation reagents. These specimens can be

stored at room temperature for up to one week, at 4 °C for up to one month, and indefinitely at –80 °C. Notably, the tissue needs to be soaked in these reagents overnight at 4 °C before being stored at lower temperatures. The following considerations for the storage and transportation of samples apply to all *in vivo* omics investigations to preserve the integrity of the biomolecules.

5.4.1 Summary of general considerations relevant to in vivo sample storage

1. The long-term integrity of the biomolecules can be maintained by storing the samples at –80 °C. Users should ensure that they have access to appropriate facilities to achieve this.
2. Careful consideration should be given to sample labelling, tracking and documenting information about the storage conditions throughout the life of the sample.
3. Labels should be recognisable and durable at –80 °C.
4. Labels should have unique sample identifiers.
5. Freeze-thaw cycles should be avoided. However, when these are essential and cannot be avoided, they should be consistent across all samples and well-documented. Users may consider aliquoting the samples prior to storage.
6. Report the following information when the samples are stored following the guidance in the OORF (OECD, 2023a) and summarised in Table 4:
7. Document the temperature and duration of sample storage (Table 4; 2.6.8).
8. Where relevant, record any processes applied to the samples during the storage period, such as the number of freeze-thaw cycles that samples underwent (Table 4; 2.6.8).

5.4.2 Summary of general considerations relevant to in vivo sample transport

1. Particular attention should be given to the consistency of the appropriate sample temperature throughout shipping to preserve sample integrity. Sealed sample tubes should be shipped on dry ice or within a dry shipper charged with liquid nitrogen.
    - Ensure that there is a sufficient volume of refrigerant to hold a consistent temperature for at least three days when shipping can be achieved within one day.
    - Plan the shipment to avoid delivery near weekends and holidays.
2. Select the appropriate packaging that maintains environmental conditions and prevents damage to the samples.
    - Sample vessels should be double-contained in sealed bags rated for low temperatures.
3. Users should pay close attention to correctly labelling packages and completing all necessary paperwork (e.g., customs-related) to ensure minimal shipping delays.
    - Retain all documents in the study files indicating date, time and signature(s).
    - Notify the receiver at least 24 hours before the planned shipment, with the applicable tracking number; send the receiver an electronic version of the inventory list.
4. Report the following information when the samples are transported following the guidance in the OORF (OECD, 2023a) and summarised in Table 4:
    - Provide a description of the method of transport (Table 4; 2.6.8).
    - Record any instances of thawing during transport (Table 4; 2.6.8).

6 Guidance on sampling whole-organism alternative test species

Among the three sample types considered in this Guidance Document, whole organism protocols uniquely provide omics data of systemic toxicity that reflects interactions between cells and organs. These interactions, which govern many biological processes, are not captured by *in vitro* and *in vivo* tissue samples. Whole organism protocols may also capture toxicity being expressed across the full diversity of cell and tissue types. New estimates for the number of cells in adults and children, drawing on over 1500 scientific papers, count 400 major cell types across 60 tissues (Hatton *et al.*, 2023). This need for whole organism testing, combined with the need for 3Rs methods, has encouraged protocols involving non-sentient model organisms. In toxicology, these are termed alternative test species.

As living organisms, alternative test species samples will differ according to their life histories. These include sex, stage of development and phenotypic plasticity (environmentally induced physiologies and phenotypes) that may introduce confounding variables to the omics data. Care should be taken to collect samples that are highly synchronised according to their life histories.

Some tissues from an alternative test species may also be easier to process than others (e.g., the toughness of the cuticle), and the diversity of tissue types is a function of taxonomy. Other modifications in the handling and processing of biological specimens may be necessary to capture biomolecular signatures of systemic toxicity representing all tissue types.

There are over 40 OECD recommended alternative test species for toxicity testing. A growing number of these are supported by omics knowledge and resources from a significant user community, particularly since the completion of the *Danio rerio* (Howe *et al.*, 2013) and *Daphnia pulex* (Colbourne et al., 2011) draft genome sequencing projects. A reference genome for model species is often a prerequisite for omics applications.

This document provides general guidance based on the biological characteristics of two distantly related alternative test species, zebrafish embryos and *Daphnia*, which serve as omics models for environmental toxicology. These species represent key taxonomic groups, with zebrafish (a vertebrate) serving as a model for freshwater fish and *Daphnia* (an invertebrate) representing branchiopod crustaceans.

Danio rerio (zebrafish) is a vertebrate model in biomedical research and its embryos are frequently used as an alternative test species to determine toxicity observed in fish following OECD Test Guidelines, including No. 236: Fish Embryo Acute Toxicity (FET) Test (OECD, 2013).

- *Daphnia magna* (Cladocera zooplankton) is a crustacean model species in ecological genomics used extensively in regulatory ecotoxicology to set regulatory limits on hazardous substances following OECD Test Guidelines, including No. 211: *Daphnia magna* Reproduction Test (OECD, 2012).

The following subsections detail specific considerations and example procedures for the five pre-analytical processes that may be applied when sampling whole organism alternative test species for omics analyses (Figure 6). General considerations are first presented that should be considered when designing and conducting any omics study. This is followed by example

procedures with several optional components which are intended to help guide users in producing a suitable protocol applied to their own test systems. The example procedures in this section are based upon studies using zebrafish embryos in multi-well plates.

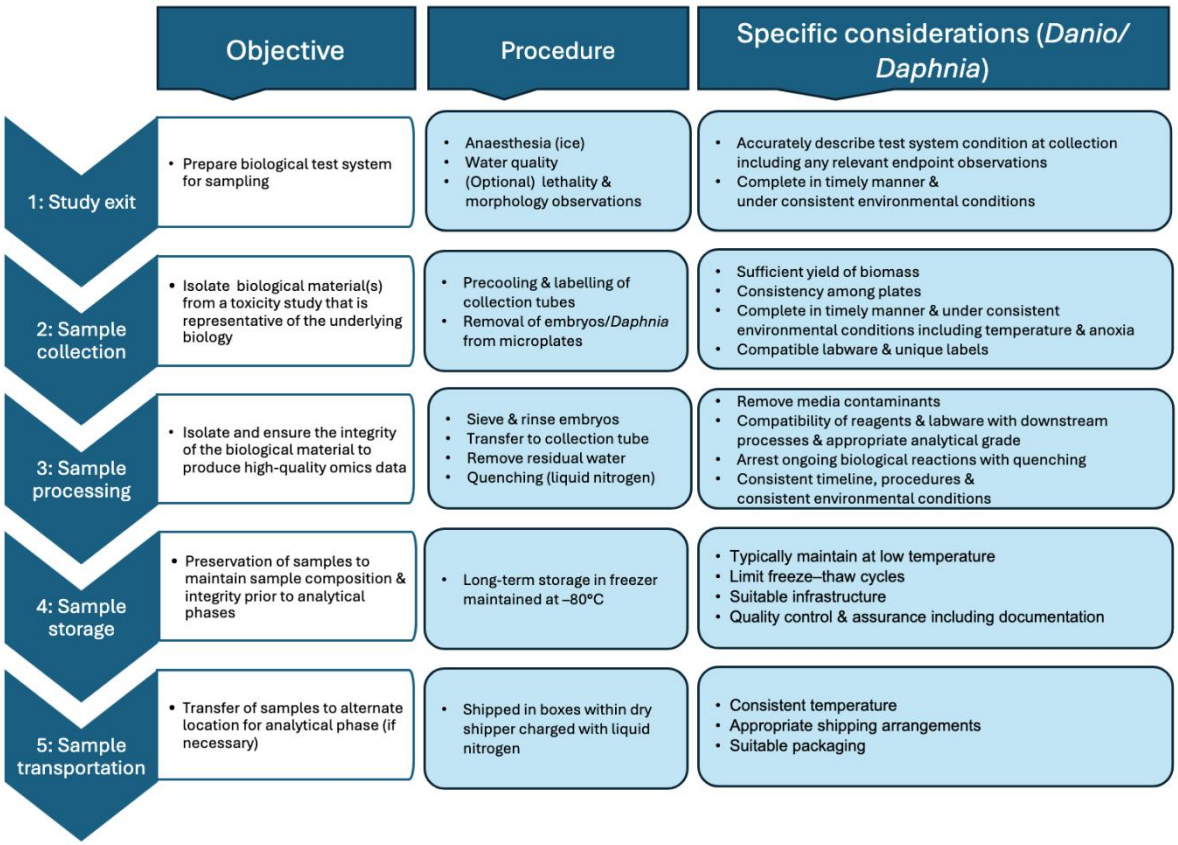

Figure 6. Main objectives, recommended procedures and specific considerations of the five pre-analytical processes used during sampling of zebrafish embryos and Daphnia from multi-well plates for omics analyses.

6.1 Study exit

The exit procedures for studies involving alternative test species typically require (1) removal of the culture vessel from the experimental environment, (2) appropriate anaesthesia or euthanasia of the test organisms, and (3) an assessment of morphological and/or apical endpoints. In contrast to *in vivo* studies, alternative test species provide higher throughput, thus reducing the time that is needed for study exit.

As part of the study exit of a zebrafish embryo or *Daphnia* study, it is often necessary to transfer the exposure vessels (e.g., a multi-well plate) from the established experimental conditions such as an incubator to another environment, such as a benchtop to observe and record apical endpoints and perform the sample collection steps. Due to the dynamic nature of biomolecules analysed with omics technologies, this change in environmental conditions can introduce confounding factors (Section 2.3) and have an appreciable effect on the abundance and diversity of certain biomolecules, thereby adversely impacting downstream results (Section 2.2.3). Care should therefore be taken to complete all exit procedures under consistent environmental conditions (e.g., temperature) and in a timely manner to minimise these unintentional environmental effects on the biomolecules. It is prudent to note the time when each vessel is removed from their experimental conditions and the time taken to complete all exit procedures to ensure a consistent timeline among samples. Ideally, the exposures and the exit procedures may be conducted

within the same environment, such as within a walk-in environmental chamber with controlled temperature, relative humidity and lighting.

To facilitate the handling of alternative test species for omics sampling, multi-well plates may be cooled on ice as a form of mild anaesthesia (e.g., for 5 minutes). Alternatively, embryos may be euthanised by transferring them into tubes, which are then inserted into crushed ice for longer time intervals (Annexe 2, SOP 6.01). The duration of time that is spent on ice should be consistent across all samples and among plates within a given study. The sampling of embryos within a plate should also be performed as swiftly as possible to prevent differences in time spent under cool (terminal) anaesthesia between different wells, as the differences in exposure to alternate environmental conditions such as temperature will introduce a significant source of biological response variation affecting relative biomolecule abundance. Alternatively, if using 96-well plates that limit the free movement of embryos, cooling the plate on ice may not be needed, thereby avoiding any potential cold stress (Annex 2, SOP 12.02). Handling plates individually minimises batch effects created by processing groups of plates (Annex 2, SOP 12.02-12.08; Section 2.3).

At the completion of the exposure study, it may be appropriate to take certain apical endpoint measurements or morphological observations that associate the omics data to quantifiable toxicological outcomes. These observations can provide confidence in the plausible toxicological interpretation of the omics data, thereby increasing the regulatory relevance of the conclusions that are drawn from the omics analyses, especially when these observed phenotypes are functionally linked to a toxicological endpoint or known chemical hazard endpoints (such as immobilisation). These observations also ensure that omics analyses are not conducted on organisms exposed to lethal concentrations instead of levels of toxicity that elicit biomolecular omics profiles of the chemical modes of action. Apical observations made on zebrafish embryos after 96 hours are described in OECD TG 236 (OECD, 2013). Users should consider employing separate biological replicates for taking apical endpoint measurements or morphological observations to those replicates used for omics sampling. Otherwise, users should minimise the impact of the extra time that is taken to perform these steps. Prolonged time intervals may introduce variability into stress responses, potentially affecting the quality of the downstream omics data and leading to biomolecular profiles that do not accurately represent the experimental treatment effects (Section 2.2.1).

The recommended procedures and considerations below apply to all three types of omics (transcriptomics, proteomics and metabolomics).

6.1.1 Recommended procedures

1. Record the exact time at which multi-well plates are removed from the exposure conditions and the duration of the exit procedure.
2. [Optional] Cool plate on ice (e.g., 5 minutes) to immobilise embryos.
3. [Optional] Collect apical endpoint measurements to assess lethality and other morphological endpoints, preferably using replicated samples to those used for omics sampling.
4. [Optional] Perform terminal anaesthesia by incubating embryos on ice for an extended period (Annex 2, SOP 6.01).

6.1.2 Summary of considerations

1. Describe biological test system at the time of sample collection.
2. Administer any treatments in a consistent manner.
3. Handle plates one at a time to avoid batch effects and to minimise delays.
4. Users should maintain consistency in the environmental conditions of samples including time spent under and temperature of any (terminal) anaesthesia.

5. [Optional] Apical endpoints and morphological assessments may be made to ensure omics analyses are not conducted on organisms exposed to lethal concentrations.

6.1.3 Reporting

Study exit involves accurately describing the status of the biological test system at the end of the exposure period, the condition of the samples, and the time when entering the sample collection step (Section 6.2). This includes reporting descriptors of the phenotypic observations and apical endpoints, if taken. The recommended study exit descriptors to be captured and reported for alternative test species, from the OORF (OECD, 2023a), are listed in Table 4.

- Describe the type of biological sample that will be used for omics analyses (Table 4; 2.6.1)
- Record the method(s) of anaesthesia, analgesia and/or euthanasia performed, if applicable. (Table 4; 2.6.2)

6.2 Sample collection

The sample collection for alternative test species typically requires the isolation and pooling of individual whole organisms from their exposure media to obtain sufficient sample quantity for omics analysis.

For the omics analysis, particular care is required for aquatic organisms such as zebrafish embryos and *Daphnia* to remove the exposure media, thereby limiting this source of unwanted biological (e.g., microbes) and chemical contamination that may manifest in the analytical phase, including unabsorbed test substances and media components. The removal of media can be achieved by using a Pasteur pipette to transfer the organism within a small volume onto a fine mesh sieve (Annex 2, SOP 12.03) for washing and in some cases for blotting. This is immediately followed by the sample processing steps (Section 6.3). Care should be taken to ensure that all samples are collected in a rapid and consistent manner, to prevent anoxia and minimise a stress response (that may introduce confounding omics results), ensuring that the temperature, lighting and other environmental conditions are the same throughout the collection process. Anoxic conditions are created when the embryos are anesthetised on ice to aid handling, and when the embryos are handled outside of media (Section 2.2.3). Therefore, the plates and samples should be handled individually to avoid unnecessary delays, to maintain a consistent timeline and to prevent significant batch effects. Sampling times should be recorded to indicate if any multi-well plates had significantly deviated from the collection time of others and hence produce unwanted technical variation in the data among the samples (Section 2.2.3).

Users should also consider the sample quantity requirements for downstream analytical processes, which may involve the pooling of individual samples from different wells under the same treatment to obtain sufficient yield. Contrary to *in vivo* pooling (Section 3.3), the pooling of whole organisms such as *Daphnia* or zebrafish embryos has the advantage of normalising unintentional intra-individual sources of variation (e.g., differences among sexes, genotypes, etc.). Ideally, more biological material should be collected than required for the analytical steps allowing replicated omics measurements, if necessary. Sampling a consistent number of whole organisms (e.g., alternative test species such as *Daphnia* or zebrafish embryos) prior to freezing may also be important to help to ensure the subsequent extraction of a consistent quantity per sample.

For transcriptomics, typical requirements for RNA sequencing are in the region of >100 ng of high-quality total RNA in suspension within >10 µL of RNase-free storage solution. These amounts can be obtained from single *Daphnia* or zebrafish embryos, yet it is typical to pool a few *Daphnia* or zebrafish embryos. Other low-input and/or low-tissue-quality preparation options are also available. In such instances, non-traditional downstream approaches often accompany these preparation options (e.g., TempO-Seq, 3-prime RNA-Seq).

For proteomics, the requirements differ by method, especially when sampling whole-organism alternative test species such as zebrafish embryos and *Daphnia*. In label-free quantification for shotgun (bottom-up) global proteomics, the recommended starting amount is generally 25-100 µg of total protein, which can be obtained from whole organisms depending on their developmental stage and size. For label-based quantification methods, such as isobaric tagging (e.g., TMT, iTRAQ), up to 100 µg of total protein per sample may be necessary. In cases involving fewer complex samples or highly sensitive mass spectrometers, lower amounts may suffice. For targeted proteomics techniques like selected reaction monitoring (SRM) or parallel reaction monitoring (PRM), each measurement typically requires a specific amount of total protein, which varies based on the target protein's abundance, the chosen methodology, and the number of targets. Analyses targeting post-translational modifications (PTMs), such as phosphorylation or glycosylation, often require considerably larger amounts of starting material due to the need for enrichment steps. While 20–100 µg of total protein may suffice in some cases, PTM-focused studies can demand 100–500 µg or more, depending on the specific PTM, the enrichment efficiency, the desired depth of coverage and the applied SOP.

Importantly, the recommended starting material for proteomic analyses is also strongly influenced by the choice of protein digestion strategy such as in-gel digestion, filter-aided sample preparation (FASP), or S-Trap-based methods as well as the sensitivity and resolution of the mass spectrometry platform employed. Recent advancements in mass spectrometry technologies, including systems that integrate ion mobility separation, enhanced nano-electrospray ionization, advanced quadrupole time-of-flight (Q-TOF) architectures, asymmetric trackless analysers, and segmented linear ion trap designs, have substantially improved detection limits and overall data quality. These innovations facilitate deeper proteome coverage and, in many cases, reduced sample input requirements.

For mass spectrometry metabolomics, requirements range from 1-20 mg of alternative whole animal tissue per sample.

Considering the various omics technologies, sample quantity requirements may also vary depending on the analytical platform used (Section 2.3). Users should thoroughly consider the downstream applications to ensure that sufficient material is collected. It is recommended that the analytics provider is consulted for recommendations

6.2.1 Recommended procedure

1. Process multi-well plates one at a time. Record the start time of the sample collection for each plate.
2. Transfer the aquatic organisms from the multi-well plate onto a sieve using, for example, a Pasteur pipette to remove the exposure media and proceed immediately to the sample processing steps (Section 6.3). An ideal estimate of sampling time is less than 1 minute per individual to prevent anoxia and/or minimise a stress response.
3. [Optional] Pool individual organisms from multiple wells exposed to the same treatment to provide sufficient sample quantity for each omics sample.

6.2.2 Summary of considerations

1. Reduce potential sources of chemical and biological contaminants by removing the exposure media during sample collection.
2. Maintain consistent environmental conditions throughout sampling, especially minimising anoxic conditions if cold (terminal) anaesthesia is applied.
3. Implement a rapid and consistent timeline for the collection of all samples. Handle plates one at a time to avoid batch effects and to minimise delays.

4. Users should ensure that enough material for omics analysis is collected (ideally more than what is required for the analytical steps).
5. Appropriate grade reagents and labware should be used. If uncertain, contact the analytics provider for advice.
6. Care should be taken to maintain consistent labelling and adherence to SOPs.

6.2.3 Reporting

Sample collection also includes reporting the procedures that are used and documenting recommended descriptors that are outlined in the OORF (OECD, 2023a) and listed in Table 4.

- Describe the methods that were used for the collection of biological sample(s) (Table 4; 2.6.2-2.6.3).
- Provide details of the type of sampling vials or tubes that are used (which should ideally be from the same batch/lot number (Table 4; 2.6.4).
- Thoroughly document the sample labels (which should be unique to each sample).
- Where applicable, describe the procedures covering the pooling or aliquoting of samples (Table 4; 2.6.7).

6.3 Sample processing

The processing procedures for alternative test species typically include (1) washing the sample to ensure that residual exposure media is removed and (2) quenching the sample.

Whole organism samples should be thoroughly washed using a fine mesh sieve (Annex 2, SOP 12.04) and in some cases blotted to remove as much exposure media as possible. Contaminants in exposure media, for example solvents (such as DMSO) that are required to achieve solubility of some test substances in aqueous solution, may interfere with downstream analysis (Section 2.2.4). Care should be taken to ensure that all reagents, including the wash solutions, are compatible with the intended downstream applications. In addition, users should minimise any transfer of wash solution into the sample collection tubes because the increased aqueous volumes can in some cases interfere with downstream extraction steps, for example disrupting solvent ratios.

Care should be taken to ensure that all the sample processing steps are performed in a systematic manner across all samples to prevent altering the exposure induced omics profile or interfering with downstream analyses. Users should consider the consistency of the wash steps, performing them for the same duration and under the same environmental conditions. Sample collection vessels must be appropriately labelled with unique identifiers using ink and/or labels that are compatible with downstream processes (e.g., suitable for use within liquid nitrogen if this will be used in later stages). Since whole organism tissues will ideally proceed through to the analytical phases (i.e., extraction) with minimal additional processing, the samples should be collected in tubes suitable for the downstream processes (i.e., DNase and RNase free for transcriptomics and/or with reduced protein binding for proteomics). For example, Precellys lysing kits contain ceramic beads for use in homogenisers thereby eliminating the need to thaw or transfer samples prior to their extraction (Annex 2, SOP 12.05). It is recommended that the collection tubes and wash reagents be pre-chilled (e.g., 1 hour on ice) prior to their use.

Quenching of the sample by flash-freezing in liquid nitrogen is then required to limit ongoing (bio)chemical reactions and maintain sample integrity (Annex 2, SOP 12.07). Rapid and effective quenching by flash-freezing in liquid nitrogen is particularly important for alternative test species such as *Daphnia* that are particularly rich in endonucleases. Overall, the sample processing steps should be completed within a limited amount of time to prevent further enzymatic activity and unwanted degradation of the samples.

For transcriptomics analysis, RNA stabilisation reagents (also known as RNA stabilisers) can be used for quenching (Section 3.4). These commercially available reagents prevent RNA degradation from occurring and they preserve the RNA integrity for longer periods of time, especially when using warmer temperatures for cryopreservation. However, for larger samples, ensuring full penetration of the stabiliser is critical, as insufficient diffusion may leave inner tissue regions unprotected, leading to RNA degradation during thawing. Homogenisation or mechanical disruption may be necessary for larger tissue samples. Users are advised to follow the manufacture's instructions for optimal results.

The recommended procedures and considerations below apply to all three types of omics (transcriptomics, proteomics and metabolomics).

6.3.1 Recommended procedure

1. Record time of sampling using a timer; the duration between removal from test vessel and submersion in liquid nitrogen should not exceed 1 minute.
2. Rinse the whole organism samples by placing the sieve in clean, pre-cooled (1 hour on wet ice) media (e.g., beaker of zebrafish embryo E3 media) for 10 seconds (Annex 2, SOP 12.04).
3. Rapidly transfer the sample to a uniquely labelled collection tube, for example using a Pasteur pipette.
4. Remove any residual media/wash solution from the collection tube, for example using a micropipette.
5. Rapidly flash-freeze by placing the collection tube in liquid nitrogen. [Optional, if transcriptomics] Preserve the tissue sample in an RNA stabilisation reagent.

6.3.2 Summary of considerations

1. The whole-organism sample should be washed to remove all the culture medium that may contain solvents (such as low concentrations of DMSO used as a carrier) and any biological contaminants such as microbes.
2. The appropriate grade wash solutions, reagents and labware (e.g., sample tubes or plates) should be used consistently throughout the sample processing steps.
3. Rapid quenching in liquid nitrogen (ideally) or dry ice is necessary for most sample types to arrest enzymatic and cellular processes.
4. Care should be taken to limit the duration of processing steps, often at lower than ambient temperature, to help maintain sample integrity and ensure consistency throughout.
5. Consistent environmental conditions should be maintained throughout, including time of collection with particular attention to temperature.

6.3.3 Reporting

Thorough documentation and reporting of sample processing steps are recommended to ensure reproducibility of the study and to facilitate the assessment and reliability of the omics data, including the recording of possible sources of unwanted variation that may affect data quality. Example reporting elements outlined in the OORF guidance (OECD, 2023a) include:

- Describe the washing procedure, including the solvents used (Table 4; 2.6.5).
- Report the quenching procedure, including temperature (Table 4; 2.6.6).
- If applicable, describe any pooling or aliquoting of samples (Table 4; 2.6.7).

6.4 Sample storage and transportation

The omics analytical steps (Figure 1) are often performed within facilities that are located at different institutions than where the pre-analytical steps are conducted, and often at later dates (up to several years post-collection). The following considerations for the storage and transportation of samples apply to all alternative test species omics investigations to preserve the integrity of the biomolecules.

6.4.1 Summary of general considerations relevant to sample storage

1. The long-term integrity of the biomolecules can be maintained by storing the samples at –80 °C (Annex 2, SOP 12.10). Users should ensure that they have access to appropriate facilities to achieve this.
2. Careful consideration should be given to sample labelling, tracking and documenting information about the storage conditions throughout the sample's life.
3. Freeze-thaw cycles should be avoided, but when these are essential, they should be consistent across all samples and well documented. Users may consider aliquoting the samples prior to storage.
4. Appropriate sample labelling, tracking and information storage should be maintained.
5. It is advisable to report the following conditions when the samples are stored and transported following the guidance within the OORF (OECD, 2023a) and summarised in Table 4:
    - Document the temperature and duration of sample storage (Table 4; 2.6.8).
    - Where relevant, record any processes applied to the samples during the storage period, including the number of freeze-thaw cycles that samples are exposed to (Table 4; 2.6.8).

6.4.2 Summary of general considerations relevant to sample transport

1. Particular attention should be given to the consistency of the appropriate sample temperature throughout shipping to preserve sample integrity. Sealed plates or sample tubes should be shipped on dry ice.
    - Ensure that there is sufficient volume of refrigerant to hold a consistent temperature for at least three days when shipping can be achieved within one day.
    - Plan the shipment to avoid delivery near weekends and holidays.
2. Select the appropriate packaging that maintains environmental conditions and prevents damage to the samples.
    - Sample vessels should be double contained in sealed bags rated for low temperatures.
3. Users should pay close attention to correctly labelling packages and completing all necessary paperwork (e.g., customs-related) to ensure minimal shipping delays.
    - Retain all documents indicating date, time and signature(s) in the study files.
    - Notify the receiver at least 24 hours in advance of the planned shipment, with applicable tracking number; send the receiver an electronic version of the inventory list.
4. Do the following based upon the latest version of the OORF (OECD, 2023a) (Table 4; 2.6.8):
    - Provide a description of the method, temperature and duration of transportation (Table 4; 2.6.8).
    - Record any instances of thawing during transport or any other instance that may have compromised the samples (Table 4; 2.6.8).

7 Recommended documentation

The collection of appropriate, comprehensive sample metadata and thorough study documentation are essential to the success of omics studies. Sample traceability should be ensured by an agreement between the facility carrying out the exposures and the users of the samples concerning metadata that may support the analytical and post-analytical phases (Figure 1). This includes details of potential detrimental conditions the samples may have experienced, affecting subsequent analysis (e.g., exposure to high ambient temperatures or repeated freeze/thawing cycles). Quality assessment should be enabled by documenting that samples are appropriately collected to proceed for further biomolecular analysis, thus providing assurance on the reliability of biological conclusions drawn from analysed data. This is particularly relevant in regulatory studies. These considerations require thorough documentation, typically captured in the Study Protocol, Study Report, Guidance Documents and any related Standard Operating Procedures (SOPs) that might provide methodological details of key aspects of the study. At the end of regulatory studies, metadata should be readily available to the end-users in the regulatory community for review.

Metadata are the supplementary information that provide context, details, and structure to the primary data collected during a toxicological experiment. This helps in understanding, interpreting, and reproducing the experimental results. In omics-enhanced toxicology studies, the extraction of metadata from the Study Protocol, Study Report and SOPs at the laboratory conducting the study is a key step towards conducting and interpreting an omics study.

When omics data will be generated from samples collected in a toxicology study, it is recommended to extract relevant information about the samples into a sample metadata table (Figure 7) during the in-life phase of the study at the laboratory conducting the study. This is particularly important to ensure accuracy during the following steps:

- Bioinformatics will be performed on the omics data;
- Sampling, data generation or data analysis are undertaken by different organisations;
- Provisional sampling is conducted for long-term storage and potential future data generation.

By providing the sample metadata table along with the study protocol and report, the toxicology study experts can be assured that the samples from the study together with their observations by laboratory staff will be correctly handled in subsequent processes.

The sample metadata table serves multiple purposes:

- Source of information for sample labelling;
- Sample storage or shipping manifest;
- Sample metadata for data generation (analytical);
- Sample metadata input for bioinformatics.

In addition to study-level information (e.g., test substance identifier, cell seeding density), the sample metadata table should also contain individual sample-level information, such as deviations from protocol (e.g., haemolysis of plasma sample, longer time to freeze) or other observations (e.g., tissue discolouration). These experimental observations are extremely valuable at the data curation and data interpretation stages.

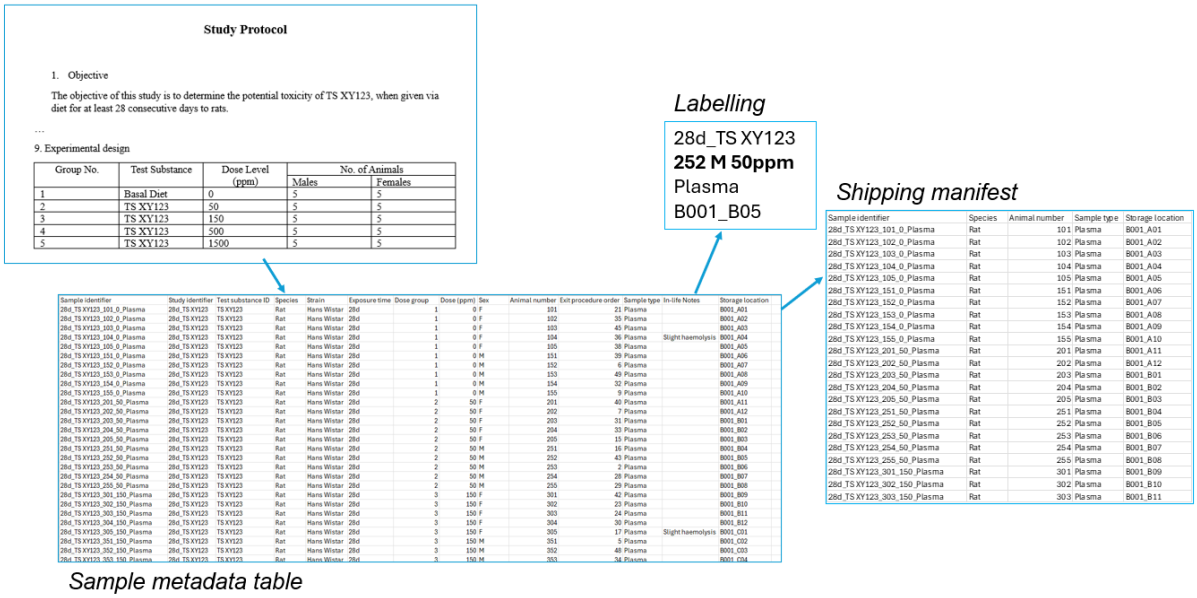

Figure 7. An example sample metadata table, which delivers information in a simple, yet structured format that can be adapted and/or filtered for various uses, such as printing cryopreservation compatible labels on a label printer, generating a shipping manifest or the instrumental analysis sequence files.

Standardised metadata fields and naming conventions will make the sample metadata tables comparable from study-to-study and enable digital tool development (e.g., for automated generation of the sample metadata table) whether within an organisation or across the wider toxicology omics community. Use of controlled vocabulary and ontologies are recommended where possible (Foster *et al.*, 2024).

An important element to consider is the *selection* of metadata fields to be included in the sample metadata table, noting that its purpose is *not* to replace the Study Protocol or the Study Report itself. The selection of metadata fields should be customised based on the study type (*in vivo*, *in vitro*, alternative test species) and ideally developed jointly by the laboratory conducting the study and omics facility.

Reporting of full technical details also promotes reproducibility and, therefore, increases the opportunities for inter-study comparisons and collaboration, in addition to potential data re-use (e.g., in the context of a knowledge base). Ensuring efficient documentation requires standardisation of reporting, which has been a focus of the OECD Omics Reporting Framework (OORF; (OECD, 2023a)).

The OORF provides modular reporting elements covering study summary, toxicological experimental details, data acquisition, processing, and analysis of omics-based toxicological studies for regulatory use, which users should consult for specific reporting fields alongside this current document. The relevant fields of the OORF, which refer to reporting sample collection, are predominantly contained within the Toxicology Experiment Reporting Module (TERM).

The TERM "*serves to capture and report the key descriptors of the* in vivo *or* in vitro *toxicology study from which samples are derived for the omics analyses".* This module contains various fields, including (OORF 2.1) Study Rationale, (OORF 2.2) Test and Control Items, (OORF 2.3) Test System Characteristics (OORF 2.4) Study Design and (OORF 2.5) Treatment Conditions, which provide relevant comprehensive guidance on the reporting of omics studies but are beyond the scope of the current document (Section 1.3).

The primary TERM users of this document should consider including 2.6 Study Exit & Sample Collection and 2.7 Sample Identification Codes. Metadata entry fields for these two sections from the current OORF version (OECD, 2023a) are reproduced in Table 4. Example entries for 2.6.1 are: 'murine left kidney', 'HL-

60 cells', 'zebrafish embryo 48 hours post-fertilisation'. Additional sources of guidance and recommendations for the appropriate fields required when reporting omics-based toxicological studies include GLP toxicology reports, OECD Harmonised Templates (OHTs) and reporting required in individual OECD Test Guidelines. However, especially for GLP studies, double reporting of data, e.g., from classical read-outs should be avoided, since GLP requires that all relevant data of a study should be reported within one report. To avoid inconsistencies, reporting of the same data in different reports is to be prevented. Consequently, if omics data for a GLP study are reported in a separate report, it has to be avoided that data from the GLP study itself are part of this report (i.e., the TERM). Nevertheless, even if omics data are included in the report of a GLP study, the omics reporting should follow the recommendations of the OORF.

Users are encouraged to consult the OORF for specific reporting fields alongside the recommendations made within this Guidance Document.

Table 4. Relevant sample metadata fields selected from the OECD Omics Reporting Framework (OORF) reporting template (OECD, 2023a).

2.6. Study Exit and Sample Collection	**2.6.1. Type of biological sample**
	2.6.2. Study exit (*in vivo*)
	a. Anaesthetic used: substance (e.g. isoflurane, ether), dosage, route of administration
	b. Analgesic used: substance, dosage, route of administration
	c. Method of euthanasia
	d. Phenotypic characteristics
	e. Methods used for collection of biological sample(s)
	2.6.3. Study exit (*in vitro*)
	a. Collection of biological material: method used (e.g. detergent), substance, concentration, duration
	b. Cell density at time of harvesting
	c. Growth phase/stage
	d. Number of culture passage
	e. Morphology
	f. Methods used for collection of biological sample(s)
	2.6.4. Sampling vial
	a. Type of vial or tube
	b. Chemicals within the sample tube (EDTA, heparins, etc.)
	c. Chemicals added to preserve sample(s) (nitrogen, argon, etc.)
	2.6.5. Washing
	a. Washing solvent(s)
	b. Washing procedure (including temperature)
	2.6.6. Quenching
	a. Quench solvent
	b. Quenching procedure (including temperature)

2.6. Study Exit and Sample Collection (continued)	**2.6.7. Pooling (or aliquoting) of samples**
	a. Describe any sample pooling procedures
	2.6.8. Sample storage and transport
	a. Post sample collection handling, prior to sample extraction.
	b. Storage temperature and duration
	c. Transportation method (e.g., between experimental facilities)
	d. Number of freeze-thaw cycles
	2.6.9. Timetable
	a. Treatments
	b. Sample collections
	c. Time since last dose administered
	d. Time to sample extractions
2.7. Sample Identification Codes	**2.7.1. Laboratory information management system (LIMS), if applicable**
	2.7.2. Method/schema for assigning unique sample codes
	2.7.3. Metadata
2.8. Supporting Data Streams	**2.8.1. Describe any supporting sources of data or information for the study (e.g. cytotoxicity assessment, pilot studies, matched apical data, etc.)**

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Annex 1. Complete list of a set of example references demonstrating the types of omics technologies and their various applications

Table of literature submitted by OECD omics sampling group experts. Key: Tx, Px and Mx refer to transcriptomics, proteomics and metabolomics. Applications include deriving a point of departure (PoD), chemical grouping (G) and mode-of-action analysis (MoA).

Reference	Omics technology			Application			Test System
	Tx	Px	Mx	PoD	G	MoA	
(Bundy *et al.*, 2024)	X			X			*in vitro*
(Bannuscher *et al.*, 2020)		X	X		X		
(Crizer *et al.*, 2021)	X		X	X			
(Malinowska *et al.*, 2023)			X	X			
(Marable *et al.*, 2022)	X		X			X	
(Ramirez *et al.*, 2018)			X			X	
(Ramirez-Hincapie *et al.*, 2023)			X	X		X	
(Rempel *et al.*, 2015)	X				X	X	
(Drake *et al.*, 2023)	X				X		
(Li *et al.*, 2023)	X	X		X			
(Vrijenhoek *et al.*, 2022)	X				X	X	
(Canzler *et al.*, 2025)	X	X	X			X	*in vivo*
(Mezencev and Auerbach, 2021)	X					X	
(Viant *et al.*, 2024a)			X		X		
(Zhou *et al.*, 2017)	X			X			
(Bhat *et al.*, 2013)	X			X		X	
(Johnson, Auerbach and Costa, 2020)	X			X			
(Lake *et al.*, 2016)	X			X			
(Mattes *et al.*, 2013)			X	X			
(Thomas *et al.*, 2013)	X			X			
(Thomas *et al.*, 2011)	X			X			
(van Ravenzwaay *et al.*, 2016)			X		X		
(Bianchi *et al.*, 2021)	X			X			
(Chepelev *et al.*, 2017)	X					X	
(Gwinn *et al.*, 2020)	X			X			
(van Ravenzwaay *et al.*, 2014)			X	X			

(Page-Lariviere, Crump and O'Brien, 2019)	X			X			
(Karkossa *et al.*, 2021)		X	X			X	*in vitro and in vivo*
(Moffat *et al.*, 2015)	X			X		X	
(Villeneuve *et al.*, 2024)	X			X			*Alternate test species*
(Essfeld *et al.*, 2024)	X			X			
(Ayobahan *et al.*, 2023)	X	X				X	
(Ayobahan *et al.*, 2019)		X			X		
(Bakker *et al.*, 2023)	X	X				X	
(Faugere *et al.*, 2020)	X	X				X	
(Gruszczynska *et al.*, 2024)	X		X		X		

Annex 2. Examples of standard operating procedures

Annex 2 contains a series of example standard operating procedures (SOPs) obtained from a community of experts and reproduced here, either in whole or in part. If shown in part, only those steps specifically associated with sampling have been included here, and other steps (e.g., related to the extraction of samples) have been omitted. The SOPs are provided, at the request of OECD Member countries, as illustrative examples of laboratory procedures associated with sample collection for omics analysis. In many cases, the SOPs include products identified by tradenames, however, the mention of a trade marked product does not indicate OECD support and other similar products may be used. In addition, the SOPs provided are not exhaustive regarding acceptable methods for sample collection, nor have the SOPs included been reviewed by OECD.

SOP Table of Contents

1. Sampling rat tissues for RNA

1.01 Optional blood samples may be collected at a specific time interval (e.g., 2 hours) following the first dose to provide estimates of toxicokinetic properties for certain chemicals.

1.02 Treated and control animals are necropsied approximately 24 hours after the last exposure.

1.03 Carbon dioxide asphyxiation is used as the method of euthanasia, with death confirmed by a secondary method such as exsanguination or cervical dislocation.

1.04 At the time of necropsy, blood is collected using potassium ethylenediaminetetraacetic acid (EDTA) as an anticoagulant via cardiac puncture.

1.05 Following collection, plasma is isolated and stored at approximately –80 °C.

1.06 While previous studies have demonstrated that transcriptional responses from the liver and kidney could be used as sentinels for phenotypic responses in other tissues (Auerbach *et al.*, 2024), a larger number of tissues will be dissected to increase the breadth of biological responses evaluated.

1.07 The dissected tissues will include kidney, liver, adrenal gland, brain, heart, lung, ovary (females), spleen, testis (males), thyroid, thymus, and uterus (females). Tissue samples are typically collected within ten minutes of termination.

1.08 The left liver lobe, right kidney, left lung, both testis, uterus, heart, spleen, thymus, and whole brain are sectioned into 5 mm^3 pieces.

1.09 Samples from these larger tissues are then individually divided into at least three cryovials.

1.10 At least two of the samples from each tissue in each animal are preserved in RNA stabilisation and storage reagent (e.g., RNALater) at 4 °C overnight and then frozen at approximately –20 °C for up to 3 weeks before transferring to approximately –80 °C.

1.11 At least one sample from each larger tissue is frozen immediately in liquid nitrogen and stored at approximately –80 °C.

1.12 The smaller bilateral tissues: adrenal glands, thyroid gland, and ovaries (female) are placed into two cryovials and preserved in RNA stabilisation and storage reagent (e.g., RNALater) with the left side of the tissue or gland going into the first cryovial and the right side going into the second cryovial.

1.13 The first tube of RNA stabilisation and storage reagent (e.g., RNALater) preserved tissue is submitted for sequencing.

2. Sampling rat tissues for metabolites

Metabolomics Broad Profiling

2.01 Record anaesthesia (if applicable) and fasting time.

2.02 Dissect a minimum of 250 mg fresh tissue from the same anatomical site of each volunteer/patient/animal according to standard dissecting protocol.

2.03 Do not touch tissue with fingers, use tweezers.

2.04 In case of liver tissue samples: Rinse off blood from liver lobes using physiological saline (0.9% NaCl), pay attention to selecting same lobe from every animal.

2.05 NO conservation with formalin or paraffin.

2.06 Place tissue into Polypropylene collection vessel immediately after dissection.

2.07 Snap freeze tissue within 5 min after dissection in liquid nitrogen.

2.08 Store the samples at –80 °C.

2.09 Ship samples at –70 °C or below.

2.10 Consistently apply same sampling procedure for entire study.

2.11 If smaller amounts are available (e.g., from biopsies), the reduction of sample amount must be discussed prior to the experiment.

3. Sampling rat blood plasma for metabolites

3.01 Sample (plasma) requirement is 150–450 microliters per platform.

3.02 It is important to use the same sampling procedure for all animals in one study: Consistency is key.

3.03 Record the exact date and time of sampling.

3.04 Blood is taken from overnight fasted rats under anaesthesia in a randomised sequence.

3.05 Retro-bulbous venous plexus is punctured with a siliconised glass orbital pipette, and blood is collected into a disposable polypropylene beaker. Other sampling routes are possible.

3.06 From beaker 1.0 mL of blood is immediately transferred with a 1 mL pipette tip into an Eppendorf tube containing 10 µL of 10 % ethylenedinitrilotetraacetic acid disodium salt dihydrate solution (e.g., Titriplex III).

3.07 The tube is closed immediately and gently mixed 5–6 times by inverting the tube.

3.08 Blood is kept cool in ice water (4 °C) during the collection period of up to 20 min.

3.09 To separate plasma, the blood is centrifuged at 4 °C, 20,000 x g for 2 min.

3.10 Separate plasma from cruor by centrifugation (centrifuge temperature set to 18–20 °C, time 10–15 min, 1000-1500 x *g*).

3.11 0.5 mL of the supernatant plasma layer are pipetted with a 1 mL pipette tip into the second labelled centrifuge tube (e.g. Eppendorf tube) and covered with an N_2 or an argon atmosphere.

3.12 Tubes are closed with the lid, which is wrapped with laboratory film, and frozen at –80 °C within a maximum of 30 min after centrifugation.

3.13 Store the samples at –80 °C.

3.14 Ship samples at –70 °C or below.

3.15 Consistently apply same sampling procedure for entire study.

4. Sampling adherent cells for metabolites (6 well culture plates - quenching): 1-10 million eukaryotic cells

4.01 This protocol recommends analysis of both cell culture pellets and supernatants. Usually, about 1–10 million eukaryotic cells (e.g., CHO cells, hepatocytes, cancer cells) are required per profiling sample, depending on the cell type. The cell number required should be jointly clarified in advance of the study.

4.02 For supernatant samples, 1–2 mL of the undiluted solution is required per sample.

4.03 The samples should be cultivated as uniformly as possible in order to obtain most similar numbers of cells.

4.04 The protocol recommends to culture 9 parallel replicates per treatment group and analysis type (Metabolomics Broad Profiling and Energy Platform). During cultivation no substrate limitation should occur.

4.05 For cell cultivation the use of dishes with a filter membrane at the bottom (e.g., Lumox®) instead of conventional well plates are recommended. Dishes can be used with a customary 6 well plate. (The cover of 6 well plates can serve as trays.)

4.06 If dishes with a filter membrane at the bottom (e.g., Lumox®) are used supernatant volume should be 2.5 mL per sample. Higher volumes lead to droplet formation at the cover.

4.07 For Metabolomics Broad Profiling:

4.08 For cell harvest, prepare a quenching solution of 27 mL dichloromethane (DCM) / 33 mL ethanol (EtOH) (HPLC grade). (60 mL serves for approx. 80 samples.)

4.09 Pre-cool quenching solution on dry ice.

4.10 Prepare labelled polypropylene vials (e.g., 2 mL Eppendorf Safe Lock™ tubes pre-cooled in liquid nitrogen). Labels should be cold-resistant (–80 °C).

4.11 Additionally, prepare 6 well plates containing approximately 5 mL isotonic NaCl (0.9%) solution in each well (avoid PBS since it can lead to phosphate-related adducts and ion suppression in subsequent LC-MS analysis) preconditioned at 37 °C. One plate is needed for two samples. (Other clean repositories can be used as well.)

4.12 Set two dishes with a filter membrane at the bottom (e.g., Lumox®) onto the 6-well plates (one in the first well of each row).

4.13 If supernatant is to be analysed, carefully transfer 1 mL of the supernatant into an appropriately labelled tube and remove floating cells or cell debris by a quick centrifugation step at low speed (e.g., 900 rpm at 4 °C). Transfer supernatant into appropriately labelled polypropylene vial (e.g., 2 mL Eppendorf Safe Lock™ tubes) and snap freeze in liquid nitrogen. The cells on the membrane should stay covered with supernatant.

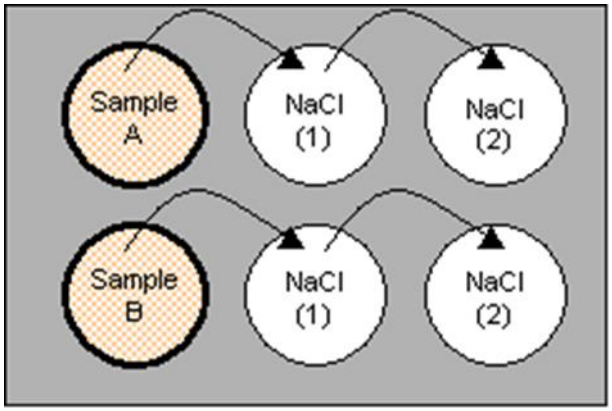
4.14 In order to remove the excessive liquid nitrogen, transfer the vials to dry ice and open lid while keeping the vial upright.

4.15 After 10 min on dry ice, close vial and wrap with laboratory film.

4.16 For cell sampling:

4.17 Cut the entire membrane out of a dish with a scalpel, so that the membrane and the supernatant fall into the washing solution.

4.18 For washing, successively dip each membrane with tweezers into the 2 washing solutions (5 mL each) in the row of the well plate (see diagram).



4.19 Use fresh plates and washing solution for each sample to avoid cross contamination.

4.20 Place the membrane cutting (with adherent cells on top) onto the pre-cooled polypropylene vial. Push the membrane into the vial by means of a pipette tip and add 600 µL of quenching solution (DCM/EtOH, –80 °C, see above).

4.21 Use fresh plates and washing solution for each sample to avoid cross-contamination.

4.22 Prepare labelled polypropylene vials pre-cooled on liquid nitrogen.

4.23 Place the membrane cutting (with the cells on the top) onto the vial and stick the membrane into the vial by means of a pipette tip and add 900 µL of quenching solution (2:1 DCM/EtOH, –80 °C, see above).

4.24 In order to remove the excessive liquid nitrogen, transfer the vials to dry ice and open the lid while keeping the vial upright.

4.25 After 10 min on dry ice, close vial and wrap with laboratory film.

4.26 Please note that the procedure from cutting the membrane until freezing should not exceed 30 seconds for each sample to prevent metabolic changes during sampling!

4.27 Store the samples at –80 °C.

4.28 Ship samples at –70 °C or below.

5. Sampling adherent cells for metabolites (96 well culture plates - quenching): 15,000 cells

For metabolomics broad profiling:

5.01 Prepare 96 well plates: cultivate ~15,000 cells (e.g., HepG2) per well in 200 µL medium. The number of initially seeded cells depends on the cell type. Ideally, 60,000–80,000 eukaryotic cells should be harvested to yield sufficient cellular mass for the metabolome analysis.

5.02 Only use inner wells of the plate (wells B2–G11) for cell cultivation (6 replicates per treatment group and 12 replicates for controls) and fill outer wells with PBS.

5.03 Cells should be harvested 72 h after seeding (e.g., HepG2). Other cell types may require a different timeline.

5.04 The samples should be cultivated as uniformly as possible in order to obtain most similar numbers of cells. During cultivation, no substrate limitation should occur.

5.05 Treatments should be applied counting the desired exposure time backwards from 72 h. For example, treatments should be applied 24 h after seeding if 48 h exposure time is desired.

5.06 If supernatant is to be analysed, carefully transfer 150 µL supernatant per well into an appropriately labelled tube and snap freeze in liquid nitrogen.

5.07 Remove remaining supernatant and wash cells with PBS.

5.08 Cover the plates with sealing mats.

5.09 Keep plates on dry ice until frozen (approx. 5 min).

5.10 Store at –80 °C.

5.11 Ship plates and (optionally) supernatant samples at –70 °C or below.

6. Sampling zebrafish embryos at 96 hours post-fertilisation for RNA and protein analysis

6.01 Following the completion of the modified fish embryo toxicity test (zFET), transfer 10 zebrafish larvae from each treatment group into individual 2 mL screw cap tubes. Carefully remove the excess medium, leaving approximately 200 µL to prevent desiccation.

6.02 Euthanise the larvae by placing the 2 mL screw cap tubes on an ice block for approximately 10 minutes. Ensure the medium remains liquid and does not freeze during this process.

6.03 After euthanasia, remove the remaining medium and add 350 µL of lysis buffer RP1 (from the RNA/Protein extraction kit) mixed with a suitable reducing agent (e.g., tris(2-carboxyethyl)phosphine (TCEP)) to each tube.

6.04 Homogenise the samples using a bead-based homogeniser at 5 m/s for 45 seconds to ensure efficient cell lysis.

6.05 Proceed with simultaneous RNA and protein extraction using the RNA/Protein extraction kit (e.g., Macherey-Nagel® NucleoSpin®) following the manufacturer’s protocol.

7. Sampling HepaRG cells for metabolites: 50,000 hepatocytes of HepaRG / well in 100 µL medium

7.01 After the time of exposure has been reached: Remove cell media from a 96-well plate and transfer the media to an empty 96-well plate (please keep media for potential future analyses by sealing the plate the same way as the plates with cells only, then freeze on dry ice and store at –80 °C - accomplish this step *after* the plates with cells are washed, sealed and placed on dry ice to freeze).

7.02 Approximately 20 µL of media will be left in a well. However, it will be removed later by a cell washer prior to the first wash.

7.03 Before dispensing 0.9% NaCl / water, the washer first aspirates the remaining volume of liquid in wells decreasing the remaining volume left in wells to approximately 5 µL.

7.04 Wash the cells immediately after the medium is removed (see table below for summary):

Wash no. and type	Volume of wash	Temperature
Wash 1: Saline (0.9% NaCl)	180 µL	4 °C / wet ice
Wash 2: Saline (0.9% NaCl)	180 µL	4 °C / wet ice
Wash 3: Water	200 µL	4 °C / wet ice

1) Wash *twice* with 180 µL of saline (0.9% NaCl).

- Cool down the saline (0.9% NaCl) in the 4 °C fridge for at least one hour prior to use. Fifteen minutes before the procedure, place the saline into a washing bottle on wet ice that is connected to the automated washer.

2) Wash *once* with 200 µL of water. The wash with water must be accomplished under 10 seconds to ensure the cells are not lysed; last time the step took approximately 7 seconds with the use of the automated washer.

- Cool down the water in the 4 °C fridge for at least one hour prior to use. Fifteen minutes before the procedure, place the water into a washing bottle on wet ice that is connected to the automated washer.

7.05 As soon as the cell washes are completed, seal the plates containing the washed cells with PCR heat sealing foil (peelable, suitable for storage at –80 °C) using a heat sealer at 180 °C for 5 seconds.

7.06 As soon as the plate is sealed, rapidly place it on dry ice for a minimum of 15 minutes.

7.07 Ensure time consistency between processing times (if possible, record the time for every plate).

7.08 Once the cell samples are frozen (after a minimum of 15 minutes), place the plates at the –80 °C freezer. Samples can be stored in this format for up to 12 months.

7.09 Ship the samples on dry ice.

8. Sampling mammalian liver for metabolites

8.01 Multiple samples of liver (4 x 150 ± 15 mg) will be taken from two 5 mm sections of the left lateral lobe. The weight of each section is to be recorded.

8.02 Each liver sample will be placed into separate 2 mL microcentrifuge tubes, compatible with downstream sample processing, and snap-frozen (e.g., in liquid nitrogen or a cryogenic mixture).

8.03 Samples should be uniquely labelled detailing the sample information (including randomised order for the lab analysis) as provided by the Sponsor. Only printed labels that are compatible with low-temperature storage are acceptable. Labels should not cover the grip neck of the vial.

8.04 A list of samples in Excel format should be shared with the Sponsor, together with relevant in-life or sampling-related observations and liver sample weights.

8.05 Samples are to be stored in a freezer set to maintain –80 °C and shipped on dry ice when transferred.

9. Terminal sampling mammalian blood for plasma metabolites

9.01 Minimum target blood volume: 0.35 mL, anticoagulant: K_2EDTA.

9.02 Minimum of 0.35 mL blood will be mixed gently and kept on crushed wet ice until centrifugation within 60 minutes of blood withdrawal.

9.03 The samples will be centrifuged at 1500 x g for 10 min at 4 °C.

9.04 The resultant plasma will be separated and 2 x 50 µL aliquots carefully pipetted into uniquely labelled 2 mL microcentrifuge tubes compatible with downstream sample processing, with the remainder to be transferred to a 3rd vial (bulk plasma sample).

9.05 Samples are to be frozen as soon as possible over dry ice or in a freezer set to maintain –80 °C.

9.06 Any evidence of haemolysis will be recorded.

9.07 Samples should be uniquely labelled detailing the sample information (including randomised order) as provided by the Sponsor. Only printed labels are acceptable that are compatible with low-temperature storage. Labels should not cover the grip neck of the vial.

9.08 A list of samples in Excel format should be shared with the Sponsor, together with relevant in-life or sampling-related observations.

9.09 Samples are to be stored in a freezer set to maintain –80 °C and shipped on dry ice when transferred.

10. Small paired organ sampling (adrenal gland, ovary) for transcriptomics

10.01 As soon as possible after necropsy, one paired organ (left) will be placed in an appropriately labelled RNAase-free cryotube**.** The remaining organ (right) is processed for histopathology.

10.02 The cryotube with tissue is snap-frozen using liquid nitrogen.

10.03 The time between necropsy and placement within liquid nitrogen is recorded.

10.04 The samples are stored in a freezer set to maintain –80 °C.

10.05 Samples are shipped on dry ice when transferred.

11. Sampling of liver, kidney, spleen, testis for transcriptomics

11.01 Liver: After weighing and sampling for histology and any other endpoints, the remaining left lateral lobe will be cut in four total sections approximately 5 x 5 x 5 mm each.

11.02 Kidney: After weighing and collection of the histology sample, the left kidney will be cut into a 2-3 mm section transversely. The renal medulla will be avoided. The renal cortex will be subsequently cut to achieve a strip of approximately 10 x 2 mm, which will be then cut into three equal pieces.

11.03 Spleen: After weighing and collection of the histology sample, the spleen will be collected into three pieces of approximately equal size (each no larger than 5x5x5 mm).

11.04 Testis: The right testis is processed for histopathology. The left testis will be cut to achieve approximately 4 equal pieces.

11.05 All tissues**:** each piece will be transferred to an appropriately labelled RNAse-free cryotube.
11.06 Each cryotube with tissue is snap-frozen using liquid nitrogen.

11.07 The time between necropsy and placement within liquid nitrogen is recorded.

11.08 The samples are stored in a freezer set to maintain –80 °C.

11.09 Samples are shipped on dry ice when transferred.

12. Sampling zebrafish embryos from 96-well plates for RNA and metabolites

12.01 Prior to sampling, transfer the beads from the required number of homogenisation tubes into a set of clean microcentrifuge tubes, then place the empty homogenisation tubes on wet ice.

12.02 Upon completion of a zebrafish embryo (ZFE) toxicity test, e.g., OECD Test Guideline 236, transfer a single 96-well plate containing ZFE from ca. 27 °C to the lab bench at room temperature.

12.03 Transfer the required number of embryos (that are needed to form one replicate sample) onto a sieve. Depending on the transcriptomics and metabolomics technologies used, 4 ZFE per sample can be sufficient.

12.04 Rinse the embryos by placing the sieve in clean, pre-cooled zebrafish embryo media (e.g. E3; on wet ice) for 10 seconds.

12.05 Using a plastic transfer pipette, transfer the ZFE as rapidly as possible into an empty homogenisation tube. Remove as much wash solution as possible from the homogenisation tube using a pipette tip and discard that solution.

12.06 Pour the beads from one of the microcentrifuge tubes back into the homogenisation tube.

12.07 Flash freeze the ZFE by placing the tube in liquid nitrogen. The time between collecting the embryos and freezing each homogenisation tube should not exceed 1 min.

12.08 Repeat the steps above to sample all of the ZFE from a single 96-well plate. Ideally the time to sample the whole well plate should be ca. 10 minutes or less.

12.09 Repeat this sampling process for all of the well plates in the study.

12.10 At the end of the sampling, store the ZFEs in homogenisation tubes at –80 °C.

Annex 3. Technical note on sampling for omics in repeated dose regulatory studies

The guiding principles for good quality sampling presented in the OECD Guidance on Good Practices and Standardisation of Sample Collection for Omics Analysis are applicable to any sample collection for omics analyses. However, if sampling is incorporated as part of a regulatory test guideline (TG) toxicity study, there may be additional practical considerations.

This technical note[1] provides practical considerations on blood and tissue sampling for transcriptomics and metabolomics, and on blood sampling for metabolomics. The feasibility of sample cryopreservation for omics, while maintaining the regulatory objective and achieving the obligatory investigations specified in the TGs (standard parameters) is a critical factor when considering sample cryopreservation. The principles, as described here, can be easily transferred to any systemic toxicity study after repeated exposure. The decision on whether to include sampling for (and subsequently to conduct) omics analysis might be made on a case-by-case basis for each study and will depend on available information on the test chemical or structurally similar chemical(s) (e.g., target organs, mode of action), the purpose of the study, as well as the needs of various regulatory authorities.

Considerations for sampling in TG studies

Blood sampling

Terminal sampling of blood for plasma collection in metabolome analysis can be considered an additional set of samples that can be obtained without significant technical or animal welfare impacts. In-life sampling of blood at one or multiple time points can bring benefits for longitudinal understanding of the development of effects or toxicokinetic data (ADME/TK), such as information on bioavailability or metabolism of the chemicals tested.

Appropriate conditions and standardisation of parameters such as anti-coagulant used, sample processing time and centrifugation steps are key for generating plasma samples of high quality for omics analysis. Please refer to the best practices and standardisation of sample collection suitable for omics analysis as described in the main document.

For sampling at the end of a study, prior to necropsy, care should be taken not to disturb or confound the standard parameters of a TG study. Therefore, additional samples (excess blood not needed for standard parameters) should only be taken if this does not interfere with the laboratory's processes and routines for blood sampling for standard parameters (e.g., haematology, clinical chemistry, hormone measurements)

[1] The note is based on Framework contract No ECHA/2022/502 report on the "General considerations in relation to the incorporation of the sample collection for omics measurements in a selected OECD TG" developed based on experience of authors and through consultations with study directors and pathologists. The relevant considerations were also discussed with the representatives from the 35 Contract Research Organisations (CRO) from EU, UK, US and India attending the ECHA CRO Days Nov 2024 (https://echa.europa.eu/-/contract-research-organisation-days).

at the end of the administration period just prior to, or as part of, the procedure for euthanasia of the animals. Only main test animals should be used for additional testing.

Based on experience, high-quality samples for metabolomics can be generated through, e.g., sublingual vein puncture, retro-bulbar puncture, heart puncture or tail vein puncture. If blood is drawn in addition to the blood used for standard parameters, samples for any optional omics analysis should only be taken after blood collection for standard parameters.

In-life sampling of blood at different time points during the study, as commonly included in (agro)chemical toxicity studies (Prior *et al.*, 2025), allows more flexibility since typically no samples are taken for standard parameters during the study. However, care should be taken to minimise the level of additional stress for the animals to a scientifically necessary minimum, for both animal welfare reasons and to avoid compromising the course of the TG study. Micro-sampling of blood has opened new opportunities for simultaneous toxicokinetic and toxicodynamic measurements using metabolomics assays, even for sample volumes ≤50 µL (Prior *et al.*, 2025).

Tissue sampling

Sampling of tissues in TG studies is more challenging as the practicality and technical viability of additional sampling must be balanced with the requirements for histopathology to ensure the regulatory validity and outcome. Therefore, tissue samples can only be taken from excess materials remaining after the prioritised sampling for histopathological evaluations or taken from organs that are not required for histopathological assessment in the TG study protocol. Only main test animals should be used for additional testing.

At termination of a TG study, organ weights are recorded before further processing of tissues. During the time between the sacrifice of the animals, the recording of the organ weights and the cryopreservation of tissue samples, the metabolome and/or transcriptome of the tissue are likely to change. However, even if some change to the metabolome and/or transcriptome occurs due to suboptimal sampling times, employing highly standardised sampling protocols with consistent timings for both control and treated animals is a viable approach to generate high-quality omics data.

The sampling conditions including excision of the organ (e.g., removing from the connective tissue), location of the organ used for sampling, and size of the tissue, should all be standardised. Please refer to the best practices and standardisation of sample collection suitable for omics analysis as described in the main document.

This technical note provides examples of what type of organs may not be used or are only partially used for histopathology purposes and could therefore be cryopreserved, e.g., for omics analysis. However, this may vary based on the requirements of different regulatory authorities and, generally, it is the responsibility of the study director and the executing laboratory to ensure that all information needed for a TG study is generated.

Examples of organs and tissues which may be available for cryopreservation as part of a repeated dose toxicity study include the following (in alphabetical order). For cases where parts of an organ could be used, the study pathologist has to be consulted on which parts could be sampled to ensure that all information needed for a TG study is generated:

- Bone marrow (femur) (if not included in TG protocol)
- Gastrointestinal tract (parts of the tract)
- Kidney (part of organ, specifically cortex)
- Liver (part of organ, specifically lobus caudatus)
- Lung (part of organ)
- Lymph nodes (except those relevant for histopathology dependent of the route of exposure)

- Pancreas (if not included in TG protocol)
- Pituitary gland (if not included in TG protocol)
- Salivary glands (if not included in TG protocol)
- Skeletal muscle (part of organ)
- Spinal cord (part of organ)
- Spleen (part of organ)
- Thymus (part of organ)
- Uterus (part of organ)

For many organs, the whole structure is required for histopathological assessment. This is typically the case for small organs, such as the thyroid gland, and for highly structured organs, such as the brain. Furthermore, for paired organs, such as adrenals, gonads or accessory sex organs, both sides may be preserved for histopathology.

Tissue sampling for omics measurements should be conducted by experienced staff trained in histological techniques who have knowledge of best practices related to omics sampling. This ensures sufficient standardisation and quality in the sampling.