Hypothesis/Commentary

Human Biospecimen Research: Experimental Protocol and Quality Control Tools

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Introduction

Among the different types of variability (interindividual, intra-individual, analytical, and preanalytical) that can influence the results of any biological assay, preanalytical variations are the most difficult to manage. Preanalytical variations are defined as any variation taking place between the moment of specimen collection and the moment of sample analysis. In a clinical laboratory setting, many errors in the accurate measurement of analyte concentrations are actually due to preanalytical variations (1, 2). Thus, preanalytical variations may also lead to incorrect results in research laboratories.

Biospecimen Research evaluates a number of preanalytical variables that can potentially impact the outcome of analytical results but are not related to inherent sample differences. Biospecimen Research is not a new discipline, and a significant amount of relevant data has already been published. Recently, the ISBER Biospecimen Science Working Group compiled publications that focus on Biospecimen Research, which can be found on the ISBER web site, http://www.isber.org/wg/bs.html. Because of the extreme variety in Biospecimen Research experimental designs, it is very difficult to draw scientific conclusions on the stability of samples and biomolecules. Therefore, defining a standard Biospecimen Research protocol would greatly facilitate the harmonization and the advancement of the field. Application of a standard Biospecimen Research protocol would allow comparisons of results obtained by different groups working on related but different approaches to the same problem, or working on the same matrices.

Biospecimen Research studies parallel the phases of development of biomarker-based screening tools, as previously described by Pepe and colleagues (3). In phase 1 ''Preclinical Exploratory,'' promising directions are identified. In phase 2 ''Clinical Assay Validation,'' the ability of the clinical assay to detect established disease is shown. In phase 3 ''Retrospective Longitudinal,'' the ability of a biomarker to detect disease before it becomes clinically evident is shown, and a rule for judging a result as ''positive'' is defined. In phase 4 ''Prospective Screening,'' the extent and characteristics of disease detected by the test and its potential false positive rate are identified.

Biospecimen research can be done within the time frame of phases 1 and 2 studies. Such Biospecimen Research can be either empirical or targeted. The goal of empirical Biospecimen Research is to assess the impact of preanalytical variation at a global level, on a profile of potential genomic or proteomic biomarkers. This Biospecimen Research study can be done at the beginning of phase 1, once the biospecimen-processing standard operating procedures (SOPs) and related acceptable preanalytical variation have been established. In this case, we need to assess the impact of the anticipated preanalytical variation at a global level, in order to be able to exclude biomarkers that are ''sensitive'' to preanalytical conditions. Such biomarkers will not be clinically suitable and it would be important to identify and exclude them during phase 1 studies. An example is provided in the section below on plasma or blood-derived cells.

Targeted Biospecimen Research will assess the robustness of a specific candidate biomarker, with presumed clinical relevance, against preanalytical variation. This Biospecimen Research study can be done at the end of phase 1 or the beginning of phase 2. The resulting knowledge allows for the optimal design of the biospecimen-processing SOPs that will be implemented in phase 3 and

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4 studies. An example is provided in the section below on urine.

An additional application of the Biospecimen Research protocol is research on the identification of ultrasensitive biomarkers that can be used as quality control (QC) tools to monitor specific preanalytical variation factors. Finally, Biospecimen Research can be applied during studies for validation of reference or quality control ''materials'' (specimens to be analyzed) in the context of any laboratory accreditation program.

Clinically relevant biomarkers can be developed when biospecimens are collected, processed, and stored according to specific and traceable SOPs (4-7). However, international standardization in uniformity of these SOPs is likely an impossible goal. Worldwide, differences in geographical settings, climates, clinical laboratory practice, clinical settings, vendors, kits, reactives, and consumables all contribute to variability. Therefore, absolute standardization of these SOPs would be difficult, potentially detrimental to the international biotechnology market, and may potentially limit technological progress. The standardization of SOPs, considered as a guarantee to the comparability of biological samples, becomes even more difficult to achieve in the context of retrospective case-control studies. Often, in this case, exact SOPs might not have been followed or may not be available in explicit details.

Biospecimen Research is necessary for a variety of reasons, including the following:

1. In existing biospecimen collections, which are used for biomarker identification and validation, the stability of a biomarker to the specific preanalytical variation factors associated with sample collection should be retrospectively tested. This testing permits confirmation that any observed associations with clinical endpoints are not due to uncontrolled preanalytical variation.
2. In prospective collections, the impact of preanalytical variations on the stability of candidate biomarkers should be tested. This concept allows researchers either to optimize SOPs for sample collection (candidate biomarker studies) or to exclude biomarkers that are significantly ''sensitive'' (labile) to preanalytical variation (''-omics'' biomarker studies).
3. In all cases, sample QC assays capable of assessing preanalytical variations should be applied. Biospecimen Research studies are also relevant here because they may provide new tools for QC purposes (8, 9).

A standard experimental protocol to evaluate the impact of preanalytical variations is presented herein by the ISBER Biospecimen Science Working Group for application in these situations. This protocol should become part of a flexible toolbox for biomarker qualification (10, 11).

Standard Biospecimen Research Experimental Protocol

The Focus of the Standard Biospecimen Research Protocol. Serum and plasma contain biomolecules (proteins, lipids, peptides, amino acids, enzymes, antibodies, cytokines, proteases, xenobiotics) that are secreted by cells, produced by metabolic routes, and modified by *in vivo* homeostatic mechanisms. Proteomic signature studies are hindered because the proteins present in blood span a range of more than eight orders of magnitude in concentration (less than 30 pg/mL to approximately 30 mg/mL) (ref. 12). In addition to the biomolecules mentioned above, serum and plasma also contain small quantities of DNA.

Other types of biological fluids may contain a more restricted set of biomolecules (e.g., urine owing to renal filtration, cerebrospinal fluid (CSF) owing to the blood-brain barrier). Sometimes, biological fluids are intended to be used in nucleic acid-based analyses or in cell-based applications. For instance, cellular immunophenotyping can be done with synovial liquid, bacteria can be isolated and cultured from urine, and viruses can be derived from serum or plasma. Among all types of analytical methods, proteomic and transcriptomic analyses are usually the most sensitive to preanalytical variations (12, 13).

It is also important to understand the inter-/intra-individual and analytical variation of the method to be used. *In vivo* preanalytical variation factors, such as the time of specimen collection, fasting conditions, the position of the patient, the patient's diet or other life habits, correspond to inter- and intra-individual variability. The standard Biospecimen Research protocol presented herein deals with the *in vitro* preanalytical variation factors. These are the factors that can be directly controlled by the clinical laboratory and/or the biorepository.

Human Subjects. The biological mechanisms underlying the impact of a specific type of preanalytical variation on a specific analyte (e.g., inactivation, proteolytic degradation, aggregation, oxidation, and apoptosis) may not be the same in healthy and diseased individuals. Furthermore, these mechanisms operate differently after death and are each affected differently by post mortem delays (14, 15). Therefore, the experimental protocol for the evaluation of the impact of each type of preanalytical variation on a given analyte by a given analytical method, should include samples from male and female subjects, who are in one of three subpopulations: ''normal,'' ''pathological,'' or ''deceased.'' In addition, the experimental protocol should include samples

Table 1. Composition of a study population in the standard biospecimen research experimental protocol

Subjects	Analytical measures		
	Low	Intermediate	High
Normal	7 male + 7 female	7 male + 7 female	7 male + 7 female
Pathological	7 male + 7 female	7 male + 7 female	7 male + 7 female
Deceased	7 male + 7 female	7 male + 7 female	7 male + 7 female

Table 2. Constant conditions to be used for serum samples

	Serum-related preanalytical conditions
Precentrifugation	Collection (primary) tube, fully filled
	Collection tube, inverted 5-10 times
	Δt coagulation, following manufacturer's instructions (30-60 min or 15-30 min)
	Collection tube, kept closed
	Collection tube maintained in vertical position
Centrifugation	No fixed angle rotor
	No recentrifugation
	2,000 × g for 10 min (or according to manufacturer's instructions)
Postcentrifugation	No inactivation (56°C)
	No storage at RT, 4°C

Abbreviations: RT, room temperature; Δt, delay.

corresponding to low, intermediate, and high analytical measures (relative to the analytical measure distribution in the population) (Table 1). Analytical measures are defined as the measures of the activity, the expression, or the concentration of an analyte or a group of analytes by a validated laboratory method (8).

The study population presented in Table 1 is a general design, which should be applicable to most situations. However, conducting studies on deceased patients is relevant only if post mortem tissues are used for research. Conducting studies in both genders may not always be necessary. Age or another *in vivo* factor (e.g., medication or diet) may be more important than gender for selected measures. At a minimum, samples from seven subjects corresponding to low analytical measures, samples from seven subjects corresponding to intermediate analytical measures, and samples from seven subjects corresponding to high analytical measures, irrespective of healthy or pathological status, should be included. Separate categories corresponding to low, intermediate, and high analytical values are relevant only in targeted Biospecimen Research, when the analyte is known. In empirical Biospecimen Research, a minimum of seven ''normal'' and seven ''pathological'' subjects should be studied.

Analysis of Samples. All aliquots should be analyzed in parallel, ideally in the same run, with the same apparatus, same calibrators, reagents from the same batch, and by the same technician. This technician should ideally be the one who has previously worked on establishing the precision of the analytical method. In Biospecimen Research, we are interested in statistically significant differences, not necessarily in clinically significant differences. Each aliquot should be tested in duplicate to provide within-run variation estimates. The precision of the method at low, intermediate, and high measures should have previously been established. If the laboratory has not previously established the method's precision, each aliquot used in this protocol should be tested six times, in order to calculate precision at low, intermediate, and high measures.

Experimental Options Based on Different Types of Biospecimens. The Biospecimen Research protocol contains standard experimental options. These options are based on NCCLS document H18-A4 (16), INCa (Institut National du Cancer, France) recommendations on the handling of liquid specimens (17-20), guidelines for preparing manuscripts describing research in clinical proteomics (21), and minimum information specification for *in situ* hybridization and immunohistochemistry experiments (MISFISHIE) standards (22). For each type of specimen, a baseline or reference procedure is indicated. The standard options correspond to both recommendations/guidelines and usual clinical practice, and also to extreme ''stress'' conditions. Options corresponding to extreme stress conditions are implemented in order to ensure detection of a preanalytical variation effect, even in the case of the more robust biomarkers.

Certain types of preanalytical variation factors will not be tested for variability, either because the variation of those factors would be irrelevant (e.g., whether a blood collection tube with no anticoagulant is completely filled or only partially filled, or freeze-thawing of solid tissue) or because it would correspond to very specific study requirements (e.g., serum inactivation at 56°C). The factors that will be held constant are defined below as ''standard conditions'' and their variability will not be tested.

For biological fluids, the different levels of preanalytical variations to be tested have been grouped into precentrifugation, centrifugation, and postcentrifugation. For solid tissues, the different levels of preanalytical variations to be tested have been grouped into prefixation, fixation, and postfixation.

Tables presented in this manuscript include the different types of preanalytical variations for serum, plasma, urine, CSF, and solid tissues, respectively. A limited, and thus ''standardized'' number of technical options or variations to be tested is stated in each table. Standardization of the Biospecimen Research experimental protocols includes standardization of these different options to be tested, so that results from different studies can be compared. For example, if the Biospecimen Research project aims at evaluating the impact of the variation factor ''time between collection and centrifugation'' for plasma, then this factor will be tested at the predefined variability levels of less than 2 hours, 2 hours, 4 hours, 8 hours, 24 hours, 48 hours, and 72 hours or at least some of these time points (but it will not be tested at 6 hours nor at 12 hours).

Serum. Table 2 shows serum-related preanalytical factors that are not expected to be tested for variation because they will be standardized in the SOP.

Table 3 shows the baseline processing of the ''reference'' serum sample. For each type of preanalytical variable, the predefined levels of variability that can be studied by Biospecimen Research scientists are indicated.

Table 3. Standard options for Biospecimen Research protocol applied to serum

	Preanalytical variable
Precentrifugation	Type of tube: serum collection tube*, serum tube with additive, micro tube with gel
	Delay time 1 (Δt1): less than 2 h*, 2 h, 4 h, 8 h, 24 h, 48 h, 72 h
	Temperature: 20°C-25°C, 3°C-7°C*, 35°C-38°C
Centrifugation	Temperature: 20°C-22°C*, 3°C-7°C
	Separator: no separator*, gel separator, nongel separator
	Number of centrifugations: 1*, 2
Postcentrifugation	Secondary container: PP tube*, plastic straw, glass vial
	Delay time 2 (Δt2): less than 2 h*, 2 h, 4 h, 8 h, 24 h, 48 h, 72 h
	Prealiquoting temperature: 22°C-25°C, 3°C-7°C*, 35°C-38°C
	Storage temperature: −20°C, −80°C*, vapor-phase liquid nitrogen
	Number of freeze-thaws: 1*, 2, 4, 10, 20, 30
	Storage duration (years): 1.5*, 3, 6, 9, 12, *n*

Abbreviations: Δt, delay; pp, polypropylene.
*Baseline processing of the ''reference'' serum sample.

Plasma. This part of the protocol applies not only to plasma, but also to blood-derived cells.

Table 4 shows plasma-related preanalytical factors that are not expected to be tested for variation because they will be standardized in the SOP.

Table 5 shows the baseline processing of the ''reference'' plasma sample. For each type of preanalytical variable, the predefined levels of variability that can be studied by a Biospecimen Research scientist are indicated.

For example, an existing collection of RNA samples that were extracted from peripheral blood mononuclear cells (PBMCs) is to be used to identify a gene expression molecular signature, characteristic of patients resistant to a specific treatment. The SOPs applied for the samples' collection and processing indicate that the time between venipuncture and isolation of PBMCs had varied between 15 minutes and 24 hours.

Therefore, an empirical Biospecimen Research study on the impact of precentrifugation delay (Δt1) may be designed to include 14 ''normal'' subjects (seven male, seven female) and 14 ''pathological'' subjects (seven male, seven female); include the necessary number of aliquots corresponding to the number of variation options (in this case, seven options of delay time: less than 2 hours, 2 hours, 4 hours, 8 hours, 24 hours, 48 hours, and 72 hours), and in order to test each point in duplicate. So, for each study subject, seven aliquots need to be prepared; define the whole preanalytical procedure to be followed. This can be either the baseline procedure (Table 5 footnote) or another procedure that will be defined among the standardized options and strictly applied to all samples. In this example, the only type of preanalytical variable that will be tested for variation is the ''precentrifugation delay.''

The statistical analysis will include analysis in each population group (paired Student's *t*) and analysis between population groups (comparison of geometric means).

In this example, the precentrifugation delay may significantly impact the expression levels of genes associated with hypoxia (*RTP801, ADM, VEGF*), T-cell function (*CX3CR1, CCR2, TIA1*), interferon (IFN) signaling (*MNDA, IRF2, GBP1*), heat shock (*HSP701A*), or early response genes (*NR4A2, DTR*) (ref. 13). In our example, these genes should be excluded from resistance to treatment-associated molecular signatures.

Urine. The urine-related standard conditions include the following:

''clean-catch'' sampling,
sterile polypropylene (PP) primary container,
centrifugation without braking,
no storage at room temperature (RT) or 4°C.

Table 6 shows the baseline processing of the ''reference'' urine sample. For each type of preanalytical variable, the predefined levels of variability that can be studied by a Biospecimen Research scientist are indicated.

For example, a collection of urine samples is to be used to monitor the production of certain cytokines, such as tumor necrosis factor α (TNFα), interleukin 6 (IL6), and leptin, following a specific therapy in order to determine the potential prognostic value. Previously published

Table 4. Constant conditions to be used for plasma samples

	Plasma-related preanalytical conditions
Precentrifugation	Collection (primary) tube, full
	Collection tube, inverted 5-10 times
	Δt contact with cells, following manufacturer's instructions (30-60 min or 15-30 min)
	Collection tube, kept closed
	Collection tube maintained in vertical position
Centrifugation	No fixed angle rotor
	2,000 × g for 10 min (or according to manufacturer's instructions)
Postcentrifugation	No storage at RT, 4°C

Abbreviations: RT, room temperature; Δt, delay.

Table 5. Standard options for Biospecimen Research protocol applied to plasma

	Preanalytical variable
Precentrifugation	Type of tube: EDTA*, heparin, ACD, citrate, sodium fluoride/sodium oxalate, micro tube with gel
	Delay time 1 (Δt1): less than 2 h*, 2 h, 4 h, 8 h, 24 h, 48 h, 72 h
	Temperature: 20°C-25°C, 3°C-7°C*, 35°C-38°C
Centrifugation	Temperature: 20°C-22°C*, 3°C-7°C
	Number of centrifugations: 1, 2*
Postcentrifugation	Secondary container: PP tube*, plastic straw, glass vial
	Delay time 2 (Δt2): <1 h*, 2 h, 4 h, 8 h, 24 h, 48 h, 72 h
	Prealiquoting temperature: 22°C-25°C, 3°C-7°C*, 35°C-38°C
	Storage temperature: −20°C, −80°C*, vapor-phase liquid nitrogen
	Number of freeze-thaws: 1*, 2, 4, 10, 20, 30
	Storage duration (years): 1.5*, 3, 6, 9, 12, *n*

Abbreviations: Δt, delay; pp, polypropylene.
*Baseline processing of the ''reference'' plasma sample.

results indicate that freeze-thawing of serum or plasma samples may have an impact on the detection of TNFα (23).

Therefore, a targeted Biospecimen Research study on the impact of freeze-thawing may be designed as follows to:

- include 42 ''normal'' subjects (14 with low TNFα values, 14 with intermediate TNFα values, 14 with high TNFα values), and 42 pathological subjects (14 with low TNFα values, 14 with intermediate TNFα values, 14 with high TNFα values);
- include the number of aliquots corresponding to the number of variation options (in this case, six options for the number of freeze-thaws are 1, 2, 4,10, 20, and 30), and to test each point in duplicate. So, for each study subject, six aliquots need to be prepared.
- define the whole preanalytical procedure to be followed. This can be either the baseline procedure or another procedure that will be defined among the standardized options and strictly applied to all samples. The only type of preanalytical variable that will be submitted to variation is the ''number of freeze-thaw cycles.''

The statistical analysis will include analysis in each population group (paired Student's *t*) and analysis between population groups (comparison of geometric means).

In this example, if a significant impact on TNFα levels is observed after two freeze-thaw cycles, appropriate SOPs for phase 3 ''retrospective longitudinal'' and/or phase 4 ''prospective screening'' studies will be implemented, specifying that samples may be thawed only once prior to analysis.

Cerebrospinal Fluid. The CSF-related standard conditions include

nontraumatic collection (clean needlestick, no hematomas),
specimen transferred directly from needle into primary container (sterile PP tube),
no transfer of the first four drops,
centrifugation without braking,
no storage at RT or 4°C.

Table 7 shows the baseline processing of the ''reference'' CSF sample. For each type of preanalytical variable, the predefined levels of variability that can be studied by a Biospecimen Research scientist are indicated.

Solid Tissue. We do not yet have a universal fixation method for solid tissues (biopsy, autopsy, surgical) that would provide optimal results for all types of end-use applications (morphology, immunohistochemistry, gene expression, DNA analysis/genotyping, and proteomic analysis). Although zinc-based fixatives or FineFix have recently been suggested as compatible with all types of

Table 6. Standard options for Biospecimen Research protocol applied to urine

	Preanalytical variable
Precentrifugation	Type of collection: first void, later void, 12 h, 24 h*
	Type of container: with protease inhibitors, without protease inhibitors*
	Delay time 1 (Δt1): less than 2 h*, 2 h, 4 h, 8 h, 24 h, 48 h
	Temperature: 20°C-25°C, 3°C-7°C*, 35°C-38°C, −80°C
Centrifugation	Temperature: 20°C-22°C*, 3°C-7°C
	Force, delay time 2 (Δt2): 1,000 × g 10 min, 2,000 × g 10 min*, 4,000 × g 10 min
	Number of centrifugations: 1, 2*
Postcentrifugation	Secondary container: PP microtube*, plastic straw, PP 5-ml tube
	Delay time 3 (Δt3): less than 2 h*, 2 h, 4 h, 8 h, 24 h, 48 h, 72 h
	Prealiquoting temperature: 22°C-25°C, 3°C-7°C*, 35°C-38°C
	Storage temperature: −20°C, −80°C*, vapor-phase liquid nitrogen
	Number of freeze-thaws: 1*, 2, 4, 10, 20, 30
	Storage duration (years): 1*, 3, 6, 9, 12, *n*

Abbreviations: Δt, delay; pp, polypropylene.
*Baseline processing of the ''reference'' urine sample.

Table 7. Standard options for Biospecimen Research protocol applied to CSF

	Preanalytical variable
Precentrifugation	Type of needle: Whitacre needle*, non-Whitacre needle Delay time 1 (Δt1): less than 2 h*, 2 h, 4 h, 8 h, 24 h, 48 h, 72 h Temperature: 20°C-25°C, 3°C-7°C*, 35°C-38°C
Centrifugation	Temperature: 20°C-22°C, 3°C-7°C* Force, delay time 2 (Δt2): 1,000 × g 10 min*, 2,000 × g 10 min, 4,000 × g 10 min Number of centrifugations: 1, 2*
Postcentrifugation	Secondary container: PP tube*, plastic straw Delay time 3 (Δt3): less than 2 h*, 2 h, 4 h, 8 h, 24 h, 48 h, 72 h Prealiquoting temperature: 22°C-25°C, 3°C-7°C*, 35°C-38°C Storage temperature: −20°C, −80°C*, vapor-phase liquid nitrogen Number of freeze-thaws: 1*, 2, 4, 10, 20, 30 Storage duration (years): 1*, 3, 6, 9, 12, *n*

Abbreviations: Δt, delay; pp, polypropylene.
*Baseline processing of the ''reference'' CSF sample.

applications (24-26), such a standardization of fixation methods in the global healthcare system is not realistic. As long as different fixation methods continue to be applied, Biospecimen Research can include standardized options for each preanalytical variation factor (Table 8). If formalin is used as a fixative, data on the buffer used must also be made available. Buffered formalin is the reference fixative.

Solid tissue-related preanalytical conditions that are expected to be standard include:

cold ischemia temperature: 22°C to 25°C, temperature from fixation to freezing, according to manufacturer's instructions (4°C for RNA later),
container: cryogenic container,
no freeze-thaws prior to analysis.

Table 8 shows the baseline processing of the ''reference'' solid tissue sample. For each type of preanalytical variation factor, the predefined levels of variability that can be studied by a Biospecimen Research scientist are indicated.

Analysis of Data. In each Biospecimen Research study, the preanalytical variation factor that is studied will be interpreted as having a significant impact if the results that correspond to aliquots having been submitted to that preanalytical variation factor exceed the limits of the analytical coefficient of variation of the method used. This calculation should be done separately for the three subpopulations studied and should correspond to low, intermediate, and high values.

Further data analyses that can be done include:

- Linear regression analysis: This analysis can be done if the coefficients of variation (CVs) between the baseline (reference) values and the values resulting from preanalytical variations are the same. It allows interpretation of the linearity between the preanalytical variation factor level and its impact on the stability of the biomarker studied.
- Deming plots or bias plots: This analysis can be done if the CVs between the baseline (reference) values and the values resulting from preanalytical variations are not the same. It allows for the interpretation of an introduction of bias due to the preanalytical variation.
- Bland-Altmann plots can be produced to show any association between the initial measure of the analyte and the impact of the preanalytical variation factor studied.
- Paired Student's *t* test: If the distribution of measures from each of the three subpopulations studied is normal, mean values can be calculated and a paired Student's *t* test can be done. This analysis shows if there is a statistically significant

Table 8. Standard options for Biospecimen Research protocol applied to solid tissues

	Type of fixation:	Preanalytical variable
Prefixation		Warm ischemia time: 0, less than or equal to 10 min*, 15-20 min, 25-30 min, 50-60 min Cold ischemia time (delay time 1 (Δt1) before fixation): 2 min, 15 min, 30 min*, 60 min, 120 min
Fixation	Cryopreservation	Snap freezing*, OCT embedding
	No cryopreservation	Buffered formalin*, methanol/chloroform/acetone-based, aldehyde-based, nonaldehyde-based/without acetic acid, nonaldehyde-based/with acetic acid
Postfixation		Delay time 2 (Δt2) from OCT embedding to freezing: 0, 15 min, 4-8 h*, 24 h, 48 h, 72 h Storage temperature (for cryopreservation): −80°C*, vapor-phase liquid nitrogen Storage duration (years): 1*, 3, 6, 9, 12, *n*

Abbreviations: Δt, delay, OCT, optimal cutting temperature.
*Baseline processing of the ''reference'' solid tissue sample.

Table 9. QC tools for samples used in proteomics, metabolomics, transcriptomics, or targeted analytical applications

Type of sample	QC tool	Scope/interpretation	Reference
Plasma	Protein S	Storage duration	31
Serum	CD40L	Exposure to RT	9
	MMP-7	30 freeze-thaw cycles	32
	Superoxide dismutase (SOD)	5-15 freeze-thaw cycles or 4 d at 37°C	F. Betsou, unpublished observations
	MMP-9	Duration of storage at −80°C	33
CSF	Amyloid protein Aβ42	Freeze-thaw or exposure to RT	34
	Truncated cystatin C	−20°C storage	35
Tissues	Vimentin	Antigenicity degradation	36
	Cyclin-D1	Antigenicity degradation	37
	Phospho-P27	Antigenicity degradation	37
	2-nitroimidazole	Tissue hypoxia	38
	pH	Tissue hypoxia	39

NOTE: References contain evidence of the susceptibility of each QC tool to the corresponding preanalytical variation on which interpretation is anticipated.
Abbreviations: MMP, matrix metalloproteinase; RT, room temperature.

difference between the baseline (reference) mean value and the mean value obtained from the preanalytical variation.

- ANOVA: If the distribution is not normal, median values (interquartile ranges) can be calculated, and Friedman repeated-measures ANOVA on ranks for pairwise comparisons can be done. This analysis allows interpretation of the statistical significance of the differences observed between two populations.
- Multiple linear regression analysis can be done if the impact of more than one preanalytical variable is studied.
- Finally, analysis of differences between subpopulations (e.g., ''normal'' versus ''pathological'') can be done through summary measurements, such as geometric means, areas under individual curves, or time required to reach a particular percentage of baseline. This type of analysis allows meaningful statistical interpretations on serial measurements that might be missed by classical *t* tests (27-30).

Biospecimen Quality Control Tools

The end-products (samples or derivatives) must be comparably fit for ''purpose,'' with the ''purpose'' being the end-use application, independent of the processing method employed.

Biospecimen Research that is done following the standard experimental protocol that has been developed by the ISBER Biospecimen Science Working Group will generate scientific knowledge on the impact of each preanalytical variation on a given assay. Once the critical preanalytical steps for an analytical application are known, researchers will only test samples satisfying those previously identified critical preanalytical conditions. In situations in which doubt exists about certain critical preanalytical conditions, quality control tests specific to those preanalytical conditions should be applied. Ideal QC biomarkers should be ubiquitous, showing an on/off response to a specific preanalytical variation and should be measurable by accessible methods (9).

Whereas international standardization of SOPs for the collection, processing, and storage of biospecimens is difficult, it is possible to standardize the SOPs for the QCs. Tables 9 and 10 present examples of endogenous or exogenous (e.g., 2-nitroimidazole) QC tools for biological samples and derivatives, respectively. These examples are based on actual state-of-the-science (31-39). For the majority of possible analytes, QC tools are not yet available and correspond to ''QC gaps.''

Conclusion

Validation of clinically relevant biomarkers should take into account the potential impact of preanalytical variation on each specific biomarker. This validation process is the focus of Biospecimen Research. Because the full study of the possible combinations of all types of preanalytical factors would require huge numbers and volumes of samples, one single research group cannot perform all the necessary testing. Establishment of a

Table 10. QC tools for molecular and cellular derivatives

Analytical application in research	Type of derivative used	QC tool
Genomics	DNA	Quantification, purity, fragmentation, cross linking
Genetics	DNA	Quantification, purity
Epigenomics	DNA	Quantification, purity, fragmentation, bisulfite conversion rate
Transcriptomics	RNA, miRNA	RIN, RT-PCR, mi-RNA qRT-PCR
Cultures, xenografts	Cell suspensions	Viability, purity
	Cell lines	DNA fingerprint
	Microorganisms	rRNA genotype

standard experimental design will thus allow scientific collaborations and comparisons of results, with each individual laboratory performing testing on part of the variation factors and part of the available options.

The benefits of Biospecimen Research based on standard options include

harmonization of Biospecimen Research and comparability of results obtained by different Biospecimen Research scientists, because the same standard options will be examined;
possibility of elaboration of a standard nomenclature of sample collection, processing, and storage procedures for biorepositories, with common codes corresponding to the different levels of variation factors;
identification and validation of new quality control biomarkers and tools for biorepositories to fill actual biospecimen ''QC gaps.''

The standard Biospecimen Research experimental protocols that are described in this document are not usually compatible with the activities of a busy routine clinical biology or pathology laboratory, because of the necessary volume of samples, as well as the required strict adherence to the analytical methods to be applied. These analytical methods may be outside the scope of a routine clinical biology laboratory, therefore, development and promotion of dedicated Biospecimen Research teams is important. These research groups can advantageously be associated with biorepositories, i.e., the facilities that collect, process, store, and provide biological samples and their cellular and molecular derivatives for use in research.

Disclosure of Potential Conflicts of Interest

F. Betsou: Biobanque de Picardie, Ownership Interest on use of CD40L, P5.

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