Before You Analyze a Human Specimen, Think Quality, Variability, and Bias

ARTICLE *in* ANALYTICAL CHEMISTRY · JANUARY 2011

Impact Factor: 5.64 · DOI: 10.1021/ac1018974 · Source: PubMed

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Published in final edited form as:
Anal Chem. 2011 January 1; 83(1): 8–13. doi:10.1021/ac1018974.

Before you analyze a human specimen, think quality, variability, and bias

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synopsis

Personalized medicine requires capabilities to detect and measure health-associated biomarkers with increasingly specific and sensitive methods, putting analytical chemists at the front lines of translational research. In their Feature article, Mark David Lim, Anthony Dickherber, and Carolyn C. Compton discuss the technical and experimental design complexities of biospecimen analysis and how analytical scientists should be involved in the experimental design process.

To eliminate the barriers between biomedical discovery and clinical application, translational research must bring together experts from different disciplines to collectively share knowledge and skill sets with the goal of improving patient care. This new paradigm for biomedical research is essential for making “personalized medicine” a reality and creates an environment for clinicians and scientists to match unmet needs with innovative approaches. Analytical scientists are essential members of these interdisciplinary teams because the vision of personalized medicine is to improve the standard of medical care by including an analysis of the patient’s molecular profile as part of the decision-making process. Unlike the current practice that depends on a clinician’s ability to diagnose using symptoms and measurement of oftentimes broadly interpretable biomarkers (such as cholesterol), this next generation of biomarkers aims to use more precise, higher resolution assays to target an individual’s biology. Ideally, this profile would contain customized information that aids in the early detection of disease, and once detected, another assay would monitor the disease’s natural course using prognostic markers. Predictive markers embedded within a device would guide the prescription of a personalized therapeutic regimen, determine proper dosing, and directly monitor efficacy of treatment in real time. Examples of combined therapeutic–diagnostic interventions that are currently on the market include Genentech’s Herceptin and Novartis’s Gleevec, which diagnose and treat patients with Her2/neu positive breast cancer and Philadelphia chromosome positive chronic myeloid leukemia, respectively.

As the source of molecules used to detect disease, the “raw material” used for clinical diagnosis is a human tissue or biofluid, generically known as a biospecimen. These

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biological materials are an observation window into a patient's health and are essential for the discovery and validation of biomarkers that serve as the foundation for most next-generation clinical technologies, including clinical and pathologic diagnostic tests, therapeutic drugs, and imaging agents.

The research discipline of the biospecimen sciences focuses on rigorously identifying the impacts of pre-analytical variability to a biospecimen's molecular composition and developing systems for annotated data to better inform the downstream researcher. As an integral component of translational research, this science aligns with the quality demands brought about by rapid advances in analytical methodologies and tools, the increased lability and smaller size of the next generation of biomarkers, and the increased resolution of data, while providing an assurance to the researcher that they are indeed comparing an apple to another apple. By validating the specific conditions that have the largest impact on data quality, a researcher can reliably determine the association between a biological marker, or panel of markers, with a health outcome by tracking and minimizing the introduction of variability and bias into the design of their experiments. This is not just limited to biomarker research; biospecimen quality is also an important aspect for validating the performance of any novel technology. Such information can be used to address issues of unreliable data from both the technology and biospecimen quality angles and help determine whether a biomarker is robust and not affected by handling procedures.

The goal of this Feature is to provide an introduction to the field to scientists who analyze biospecimens as part of their effort to advance translational medicine. Because biospecimen quality can affect the presence and levels of various biomarkers, analytical scientists must communicate their quality requirements to their clinical counterparts. Experimental design needs to take into consideration that the immediate health management of the biospecimen donor—the patient—is the priority of the clinical team and that some of the procedures used to collect the biospecimen may not be controllable, but at the very least, parameters of collection should be noted for the laboratory-based researcher. This article concludes with a brief description of U.S. and international efforts that aim to ensure that researchers have access to high-quality biospecimens. If there is a single take home message from this article, it is that laboratory-based scientists must work with biostatisticians and clinical research methods experts while planning, conducting, and interpreting their biospecimen-based experiments.

THE ANALYTICAL CHALLENGE OF BIOSPECIMENS

The demand to discover and use a next generation of biomarkers, ones that have the resolution to stratify individuals with similar symptoms into specific disease categories and treatment outcomes, has pushed the limits of analytical technologies currently available in the clinic and life sciences laboratory. The challenge is further increased with demands for improved safety, efficacy, speed, and accuracy. The "-omics" era is a direct result of this new effort and employs cutting-edge analytical approaches and technology platforms to interrogate patient specimens for health-associated biomarkers. Current examples include cytomics (whole cells), genomics (RNA/DNA), metabolomics (metabolites), peptidomics (peptides), and proteomics (proteins).

Yet, unlike an off-the-shelf neat reagent, a biospecimen contains biologically active molecules, the activities of which are designed to ensure survival of the host. These components react to chemical and environmental stresses and remain active during and after removal of the biospecimen from the body. For example, several signaling pathways become activated in response to the stress induced by ischemia and hypoxia[1] (reduction of blood and oxygen, respectively) when a tissue's blood supply has been reduced as part of a

surgical resection procedure, and once transferred from body temperature to room temperature or ice, the tissue's biology may attempt to mitigate damage caused by hypothermia. These effects aren't limited to tissues; methods used to collect and store biofluids such as urine[2] and blood[3] also affect the molecular composition of these biospecimens.

Chemical reagents are used as part of the standard-of-care to minimize the impact of these stresses, which can vary from clinic to clinic, on a collected biospecimen. Tissue specimens are "fixed" and rendered room temperature stable through the use of formalin (aqueous formaldehyde) to broadly crosslink protein amine groups, and EDTA is used to chelate calcium (a signaling molecule in the coagulation cascade) to prevent blood from clotting inside the test tube. Although these biospecimen processing techniques have been used for several decades, protocols are not commonly uniform, even within the same institution.

An often neglected consideration is the effect of variability prior to the collection of the biospecimen, which tends to be dependent on the judgment and care decisions of the clinical teams. For example, anesthesia induces a biological response such as sedation or pain relief, and variability in anesthetic type and dosage may result in different analytical results between specimens that have been similarly classified. In the same way, a patient's hydration levels and diet could affect the composition, concentration, and density of biomarkers within urine or blood samples. In addition to issues of variability, bias that is introduced at the point-of-care may affect the resulting molecular analysis so that the data cannot be objectively compared with other studies and are not representative of the broader population.

Taken as a whole, these pre-analytical responses might be "recorded" in a biospecimen's biological and chemical composition so that it is no longer completely representative of a patient's health. Stresses caused by acquisition, processing, and storage conditions add to the variability. The need to separate data reflecting the biological artifact caused by the ex vivo stresses that vary between patient samples from those that represent true biology complicates attempts to directly compare two "similar" biospecimens.[4] Because personalized medicine aims to improve patient care based on an individual's specific disease and response to therapy, variability and the potential for biased research results add additional hurdles for advancing translational medicine.[5]

Evidence supporting the effects of pre-analytical variability on the quality of molecular-scale data is emerging as scientists begin to utilize higher-resolution analytical technologies to interrogate patient biospecimens.[6–10] As one example, Issaq et al. published a comprehensive review on the use of proteomic-based technologies to analyze blood specimens.[11] Reviews such this are rare, and the majority of published studies focus on investigating the effects of ex vivo biospecimen trauma to the biomarkers targeted by classic chemistry tests used in the clinic (such as blood glucose). Although these are directed to a clinical audience and not on the reading lists of most physical and life scientists, the data and observations conceivably can be extrapolated to inform the design of modern biomedical experiments. For example, analytical scientists seeking to discover peptide or protein biomarkers in blood would benefit from reports in the hematology and transfusion sciences that describe how platelets—which release various proteases to cleave amide bonds—can become activated by the shear forces within the collection needle or by the improper handling of a collection tube. Table 1 lists a selection of review articles and scientific reports that discuss biospecimen variability by type of analytical platform.

Two companion research articles describing the use of MS to analyze serum samples for prostate cancer biomarkers highlight the importance of this type of information to the

analytical researcher.[6, 12] Previous studies suggested that prostate cancer patients can be distinguished from healthy patients with >90% accuracy using a specific MS-based technique. During an attempt to translate these preliminary results into a functional clinical assay, several sources of bias and variability in the original experiments were identified. Once researchers mitigated these sources and repeated the experiments, they concluded that this technique actually detected a signal attributed to differences in serum storage and handling, rather than discriminating between healthy and prostate cancer patients.[6, 12]

WORKING WITH BLOOD BIOSPECIMENS

Most translational research experiments that involve blood specimens use previously collected and frozen samples as the first step, without regard to reports in the clinical literature that describe how methods chosen for its collection, processing, and storage can affect molecular composition and bias analytical results (Table 2). To truly understand the lifecycle of the biospecimen, the researcher needs to consider the variability that could occur during preparation of the patient and collection and processing of the blood specimen.

Blood travels through >60,000 miles of vessels and is vital for regulating the normal and protective processes of the body. Its chemistry and composition continuously vary during transit throughout all the tissues in the body, making it a biofluid that potentially contains a wealth of health-associated biomarkers.[13] Blood is also an ideal biospecimen for diagnostics because its collection is routine, quick, and minimally invasive. Though its cellular components also may contain valuable biomarkers, blood cells are usually removed, leaving plasma or serum to be analyzed. (Plasma is the non-clotted liquid fraction of blood prepared by the addition of an anticoagulant and centrifuged to remove the cellular material. To prepare serum, the liquid portion of blood is coagulated, typically through the use of clotting agents, and the clotted portion separated via centrifugation and discarded.)

Blood is a biologically active fluid, and its diverse components retain their natural roles and reactivity in the collection tube and may continue responding to the different handling and storage stresses experienced under ex vivo conditions.[14] Though a targeted biomarker may be a peptide, protein, or nucleic acid, blood also contains several different types of cells, metals, electrolytes, metabolites, hormones, and other biomaterials that have the potential to interact with that biomarker. For example, as noted above, activated platelets utilize several proteases as part of the coagulation cascade. These active proteases can potentially interact with proteins and peptides of interest to the analytical researcher. In the clinical laboratory, pre-analytical problems such as ex vivo platelet activation account for nearly 70% of the errors encountered during the analysis of blood samples (which includes both procedure-based and technician-specific errors).[15]

As shown in Table 2, many of the ex vivo stresses on a blood biospecimen occur at early stages of biospecimen acquisition. For example, the preparation of a blood specimen into its plasma, serum, or whole blood components starts simultaneously with the collection process. Collection tubes are typically pre-loaded with chemical reagents, many of which may exhibit promiscuous reactivity toward blood components, including the targeted biomarker.[16–18] Physical forces such as centrifugation and shaking are also used to prepare the collected sample. These processing steps are example sources of variability that could complicate comparison between samples, particularly if a protocol is not standardized.

WORKING WITH TISSUE BIOSPECIMENS

Tissue is removed in various sizes and states from a patient, usually for a biopsy for diagnosis or prognosis or through a resection procedure for disease treatment. Currently, the majority of tissues are archived in hospitals and clinics in a “formalin fixed and paraffin

embedded" state (described as "FFPE tissues"). Many care centers lack the infrastructure to collect and/or store frozen samples and prefer this processing method because the tissue biospecimen is stable at room temperature.[19] Translational researchers must design and conduct their experiments so that the conditions on the research bench mimic those of the clinic, particularly if the targeted biomarker can only be isolated in frozen versus FFPE tissues.[20]

Traditionally, pathologists have used microscopy and gross characteristics (such as mass and volume) to analyze tissue biospecimens for morphologic, histologic, and immunohistochemical information. However, the new -omics based technologies for analyzing a tissue's molecular composition have increased resolution and are now capable of detecting variations in collection, handling, and processing conditions. Unlike blood biospecimens whose transition from patient to phlebotomist to biobank is relatively direct, tissues are subject to processing and handling by several clinicians before they arrive at the biobank. During the surgical procedure, anesthesiologists, nurses, and surgeons introduce different types of stress to the tissue biospecimen that alter its molecular profile from its natural state—stresses that may vary between clinical teams. The specimen is then transported outside of the sterile operating room and delivered to the pathologist after being subjected to varying temperatures and time delays. Within the pathology suite, factors such as time at room temperature, temperature of room, type of fixative, time in fixative, method and rate of freezing, and size of specimen aliquots may introduce additional artifacts until the tissue is "stabilized" and stored in a biorepository.[19] Other acquisition variables such as packaging, which may have varying desiccated or oxygenated environments, can also add to the complexity.

APPROPRIATE EXPERIMENTAL DESIGN

One approach for identifying breast cancer biomarkers might be to compare the analytical results of "cancer" versus "healthy" specimens. As an illustration of factors that must be considered during experimental design, sources of bias can include sex, medications, or age: a healthy male breast sample is inherently different than a healthy female breast sample, cancer patients are exposed to different types of medications than a healthy individual, and age may play a factor in the type of individual willing to donate a healthy breast sample. Were both sets of specimens also collected, processed, and stored under similar conditions? All of these differences may turn up as putative biomarkers that are indicative of a biased sample set rather than a simple diseased versus healthy comparison.

Until it is possible to integrate evidence-based standard operating procedures (SOPs) that equally account for both clinical care and downstream research, analytical researchers need to work more closely with other translational researchers to better design and conduct their experiments and interpret the data. Following ethical, legal, and privacy policies, translational researchers must incorporate their molecular data with details on how a biospecimen was collected, processed, and stored. Multiplexed analytical assays that use an algorithm based on data collected from a panel of biomarkers add complexity to the experimental design process because individual biomarkers are likely to react uniquely to a specific stress, producing complicated patterns of variability of biomarker presence and/or expression level. If researchers understand how a specific biomarker reacts to a specific ex vivo stress prior to the collection of the biospecimen, they can work with biostatisticians and clinical research experts to identify and control sources of variability and bias through proper experimental design. Proactively engaging these experts early will help to ensure that the biospecimen is collected and analyzed appropriately, leading to rigorous data that can be consistently reproduced.

If the researchers cannot control the sources of variability and bias prior to analysis, it is still important to have access to data on collection and handling of the specimen, and these sources of variability and bias should be properly annotated within the context of the analytical data. Larger sample sets or statistical massaging do not simplify or normalize out bias from an experiment,[5] nor does reproducibility indicate a lack of bias. In the end, additional properly designed experiments will still be necessary to validate robustness and justify the health indication of a biomarker. Although identifying these factors may seem like searching for a needle in a haystack, accounting for systematic differences that occur in steps prior to biospecimen analysis provides the researcher options for addressing causes of irreproducible data and identifying data that may be reproducible but is biased and not broadly applicable.

STANDARDIZATION THROUGH BIOBANKS AND STANDARD OPERATING PROCEDURES

Researchers typically have access to a biospecimen only after proper consent has been given and it is no longer needed for a patient's care. Health management is the priority of any clinical center, and thus, the creation of a SOP for the collection, handling, and storage of a biospecimen is not easy because it could potentially affect a clinic's standard-of-care and workflow. Groups such as the College of American Pathologists and the Clinical and Laboratory Standards Institute are trying to address this challenge, but the scientific evidence that would inform the development of these protocols is still lacking. For example, there is a dearth of data describing the physical forces imparted to solutes upon the liquid–solid transition during freezing. This basic knowledge could lead to further experiments that seek to understand how such forces affect the integrity of various biomolecules in serum or plasma and assist with the development of protocols prescribing thawing methods for these types of samples. These increments of knowledge from multiple disciplines would go a long way toward transforming the science behind proper specimen collection.

As an intermediate between clinical and research efforts, biobanks (or biorepositories) are becoming increasingly valuable resources for translational researchers. The goal of a biobank is to provide researchers access to biospecimens that have been processed and stored following a standard procedure. Analytical researchers interested in mining these biobanks should understand that there are two primary types: population-based and disease-based. Population-based biorepositories focus on the collection of normal/healthy biospecimens annotated with data describing an individual's habits, exposures, demographics, and health/disease state for the purpose of epidemiology-focused studies. While most of these biobanks support longitudinal studies focused on following health outcomes over time, many also make their specimen collections available for other research. Disease-based biorepositories, on the other hand, focus on collecting diseased and sometimes healthy/normal biospecimens.

The utility of these biobanks to an analytical researcher is dependent on the end goal of the experiment, and different experimental design issues need to be considered when choosing between one type of bank from another. Bias will always be a problem if not addressed prospectively. More data is not necessarily better, and the use of biospecimens collected for one study may not be appropriate for a different study, even though the classification (type, stage, and grade for cancer biospecimens, for example) may be similar.

A number of privately and publicly operated biobanks provide a variety of specimens for research. Of those that are publicly financed, academic centers represent the largest proportion, and many have public websites with an inventory search feature. The U.S. Government sponsors or operates hundreds of biorepositories, mostly through the National

Institutes of Health (NIH) and the Centers for Disease Control and Prevention, though two of the largest biorepositories are run by the U.S. Department of Defense. Some examples of NIH sponsored efforts include the Biologic Specimen and Data Repository Information Coordinating Center,[21] the Office of Rare Disease Research's Biospecimen Repository Database,[22] as well as those of the National Cancer Institute (NCI).[23] The NCI is also developing the Cancer Human Biobank (caHUB, http://cahub.cancer.gov), a biorepository that aims to collect high-quality and annotated human biospecimens as a public resource for the cancer research community. In addition to the type, grade, and stage of cancer, caHUB plans to provide biospecimens annotated with collection, processing, and storage conditions in compliance with the latest ethical, legal, and privacy policies.

Several international efforts also focus on biobanking, and a recent review by Vaught *et al.* provides an overview of these initiatives.[24] The biospecimen sciences is also being advanced through efforts such as the NCI Office of Biorepositories and Biospecimen Research, professional societies such as the International Society for Biological and Environmental Repositories (ISBER), and through consortia such as the SPIDIA project (http://www.spidia.eu/) or the Public Population Project in Genomics (P3G).

PERSONALIZED MEDICINE AND THE ANALYTICAL CHEMIST

Analytical chemists and scientists are valuable members of translational medicine teams, and they must participate beyond the analytical components of a project. Issues such as bias cannot be simply addressed solely by following SOPs, employing better annotation, or using larger sample sizes, and analytical researchers need to collaborate with clinical research methods experts to reduce bias and address systematic differences in the design of their experiments.[5] Analytical chemists also must be comfortable asking questions—is the experiment designed to reduce variability and bias, is the quality and source of the biospecimen appropriate to reliably determine the health indication and fitness of the biomarker, etc.—and actively participate in the design of the experiment and the interpretation of the data. This dialogue should be iterative, and analytical chemists should identify for their clinical counterparts which biospecimen handling procedures have the greatest potential to affect the quality of data. These discussions all need to be held keeping in mind that the health management of the patient donating the biospecimen is the immediate priority.

Moving the practice of medicine away from an art to a more algorithm-based analytical science requires a balanced discussion of clinical needs with the scientific tools and methods that can be created to meet those needs. If personalized medicine is to become a reality, scientists need to be comfortable working within a new team-based framework in which colleagues are experts outside of their department's silo. The work, expertise, and collaboration of these diverse teams of analysts, clinicians, informatics experts, biostatisticians, life scientists, and translational researchers provide hope of improving the life of the patient through a more personalized medicine.

Acknowledgments

The authors would like to thank Prof. David Ransohoff (University of North Carolina Chapel Hill) for his comments on this article, his continuous discussions, and willingness to be a valuable resource on the clinical research aspects of biospecimen science. M. D. L. and A. D. would also like to thank the American Association for the Advancement of Science (AAAS) for supporting both with Science and Technology Policy Fellowships at the NCI Office of Biorepositories and Biospecimen Research.

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Biographies

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Table 1

Examples of reviews and articles in the literature, by analytical platform.

Biospecimen	Biomarker Type	Refs
Blood	Proteomic	25, 26
Blood	Genomic	27–29
Urine	Proteomic	30–32
Urine	Genomic	33, 34
Tissue	Proteomic	1, 35, 36
Tissue	Genomic	19, 20, 37

Table 2

Examples of pre-analytical variables and associated molecular effects in blood specimens.

Process/procedure	Some effects of variability	Refs
Patient posture	Blood volume, biomarker density, selective isolation of specific sizes of biomarker	38–40
Venipuncture site	Composition of biomarker	41–44
Syringe/needle type	Gas permeability, platelet activation, hemolysis	16, 45–48
Tube additives (anticoagulants, etc.)	Composition of biomarkers, side-reactions between biomarkers and additive	18, 49–52
Tube fill volume	Hemolysis, concentration of tube additives	53, 54
Tourniquet duration	Blood volume, biomarker density, selective isolation of specific sizes of biomarker	55–58
Storage conditions	Platelet activation, stability of components	59–62
Freeze-thaw	Platelet activation, hemolysis, genomic and proteomic stability	63, 64