Best Practices for Sample Storage: A Report from the Workshop on Urine Biospecimen Handling

Opening Remarks and Objectives

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Introduction

Urine is highly useful as a testing matrix. It is easily accessible, can be collected noninvasively, and provides information on numerous physiological processes. Urine is a source of numerous potential biomarkers, including metabolites, cells, proteins, and nucleic acids. The establishment of best practices for urine collection and storage is particularly timely since this biological fluid is a potentially important source of biomarkers of kidney disease. The development of biomarkers to monitor the safety of drugs and manage acute and chronic kidney disease (CKD) and other renal conditions is of great interest to nephrologists and biomedical researchers. The National Institute of Diabetes, Digestive and Kidney Diseases (NIDDK) convened a workshop on *Best Practices for Sample Storage: Urine as a Paradigm* in February 2010. The goals of NIDDK's workshop were to summarize current best practices for the collection, handling, and storage of urine specimens, and to discuss how best to develop more uniform protocols for future specimen collection. Since new protocols must be relevant for many fields, clinical pathologists, basic scientists, clinical trial experts, and representatives from industry were invited to participate in the Workshop.

Optimal collection and storage of urine specimens for various biomarker discovery and validation studies remains to be determined. Many existing specimens were collected and stored using different protocols or under unspecified conditions. NIDDK is funding many cohort studies and randomized trials that are currently collecting and storing large amounts of urine samples. While existing methods are sufficient for studying traditional biomarkers such as creatinine and albumin, it is unlikely that the current hodgepodge of collection techniques will be useful for modern proteomics, metabolomic, RNA, and miRNA discovery and validation studies.

Urine as Biospecimen: Standardization, Storage Effects, Harmonization

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To be used successfully for biomarker discovery and validation, various urine specimen parameters must be harmonized, including collection method, volume collected, timing of collection, processing, and storage. Different collection methods might not be comparable, underscoring the need for accurate documentation of collection and handling procedures and, when possible, the need to avoid mixing specimens from different collections. Parameters that may vary across collections include: (1) dilution or concentration of the specimens; (2) diurnal

and other temporal variations in collection; (3) the impact of diet, medications, or activity on urine composition; and (4) use of centrifugation, which may result in loss of some analytes. Investigators interested in using specimens collected in longitudinal studies must ensure that all specimens were collected and treated in the same manner across the time of the study. Investigators must also understand how storage conditions might affect the stability of various urine components. The ability to use specimens collected in other studies will expedite biomarker validation. Collection and documentation also can be problematic, particularly when seeking Food and Drug Administration (FDA) approval for a biomarker for a particular purpose. Linkage to patient history is needed for full utility of specimens, and large studies may not provide adequate control specimens.

Possible solutions to these concerns include: (1) standardization by limiting parameter combinations, (2) upfront assessment of general analyte-type suitability for a particular collection, (3) validation for specific analytes, and (4) documentation of known variables. These can be achieved by adopting Clinical Laboratory Standards Institute (CLSI) or International Standards Organization (ISO) standards. Issues to be considered when standardizing storage include: (1) the effects of long-term storage on different analyte types; (2) potential leaching of container components into urine (e.g., bisphenol A); (3) analyte stability; (4) whether or not to aliquot the sample into smaller volumes; and (5) minimization of freeze-thaw cycles. The most commonly used container types are plastic and glass. Plastic is safe, but leaches substances into the urine and also binds some analytes, altering measurement of low-level analytes in particular. Glass does not leach into the sample, but poses a safety hazard from breakage and binds some analytes. The paradigm of blood collection tubes could be applied, where blood intended for different types of analyses is collected in specific amounts and into specific (or different) types of containers. Using this approach, urine would be aliquoted into appropriate containers for subsequent processing, storage, and analysis.

Harmonizing urine collection, handling, and storage will be critical to the discovery of biomarkers, as well as validation and clinical use. Use of specimens with comparable, documented histories during the discovery phase will be more likely to yield clinically meaningful discoveries. Such discoveries may also prove more likely to be validated. Harmonizing urine specimen collections also will simplify clinical use of biomarkers. Standardization and harmonization guidelines for urine will be most useful if they reduce the number of variables that may differ across urine specimen collections.

DISCUSSION

Documentation is particularly important for the FDA. To avoid uncertainty about the validity of the discovery, investigators must document that the specimens were obtained from well characterized patients and handled in uniform fashion.

Unfortunately, specimens collected during the course of routine clinical care are unlikely to adhere to these guidelines. Strict collection guidelines for discovery and validation may in some ways limit the clinical utility of the biomarker. In general, biomarkers used in clinical practice must be highly stable. Improvements in product

design, however, have allowed clinical validation of highly labile RNA-based biomarkers. (Collection tubes that are treated to stabilize RNA are used). Study scale also must be considered when developing guidelines, as some parameters can be adhered to carefully in smaller studies, but simpler guidelines might be needed for larger, higher power studies.

Resources and funding for specimen storage, particularly for long-term epidemiologic studies, is critical. Substudies for quality control and reproducibility should be performed early in such studies. One issue is that such studies are not high priority because few can be published in high-impact journals. FDA guidance should be sought for biomarker studies to provide perspective on issues that will be necessary for qualifying biomarkers. The Interagency Oncology Task Force has made progress in this area.

Urine Handling, Sensitivity, and Immunoassays

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The types of urine specimens typically sent to a urine immunodiagnostics laboratory include freshly voided samples (30 minutes to 2 hours), cold samples (stored at 4^{o}C for up to 2 days), frozen samples (-20^{o}C or -70^{o}C, indefinite storage), lyophilized samples (usually 4^{o}C, indefinite storage), and urine dried spots (from neonatal and pediatric patients). Immunoassays depend on tight epitope-antigen receptor interactions. Antibody-antigen reactions are noncovalent and reversible. Noncovalent forces that hold components of an immunoassay together include hydrogen bonding, electrostatic bonding, hydrophobic bonding, and Van der Waals forces. All these are affected by biological characteristics of the urine matrix, such as pH, salt concentration, auto-antibodies, bacterial infections, viscosity, and naturally occurring cross-reactive molecules. Urine storage and handling parameters (e.g., prolonged poor storage prior to testing, multiple freeze-thaw cycles, samples taken from inadequately thawed specimens, and poorly labeled samples) also can affect immunoassays.

The effect of different sample handling and storage conditions on the reliability of immunoassays has been evaluated. Urine parameters were examined for their effects on immunoassays for the cytokines IL-1β, TNF-α, and IL-6. Urine pH between 5 and 7.5 had little effect on the detection of the three cytokines. A slight decline in detection was noted at sodium concentrations between 150 and 350 mEq/L. Detection was more seriously affected at low cytokine levels (< 100 pg/mL). A decline in detection also was observed as the specific gravity of the urine increased between 1.005 and 1.025. The effects of salt removal or reduction on detection were examined. Dialysis prior to analysis improved detection by less than 10%, desalting columns improved detection by less than 5%, and centrifugal ultrafiltration and sample dilution had little effect on detection sensitivity.

Storage at room temperature for 12 hours reduced detection of IL-1β and IL-6. Storage at 4^{o}C also reduced detection of these analytes, although less so than storage at room temperature. Storage for 24 hours at room temperature or 4^{o}C reduced detection

further. All three analytes were most stable when stored at −20°C or −70°C. Particulate removal by centrifugation or ultrafiltration increased detection of all three analytes above levels detected in untreated urine. The effects of contamination by several bacterial and fungal species on detection were analyzed, and both types of contamination reduced detection.

Exposures to freeze-thaw cycles are a significant issue in the storage and use of urine specimens. Increasing numbers of freeze-thaw cycles greatly reduce detection, with the largest decrease in detection occurring after five freeze-thaw cycles. Incomplete thawing of the specimen when removing an aliquot for analysis can significantly affect detection and can result in concentration of an analyte in incompletely thawed samples. Investigators should be sure to thaw the specimen completely, even if only withdrawing a small volume for analysis. Storage techniques other than freezing also were examined. Lyophilized samples and dried spots were found to be stable and allowed accurate detection of IL-1α, IL-6, and TNF-α.

Detection sensitivity in urine samples can be improved by centrifuging and adding enzyme inhibitors to fresh or short-term storage samples. Frozen stored samples used for immunoassay assessments should be processed within 30 to 60 minutes, clarified by centrifugation, treated with enzyme inhibitors, and stored at below −70°C in small aliquots that permit only one freeze-thaw cycle.

Standard procedures for storing urine samples intended for use in immunoassays are needed because the preparation and storage of urine can affect assay sensitivity. Microorganism contamination, multiple freeze-thaw cycles, and inadequate thawing of large-volume stock specimens can have a negative effect on assay sensitivity.

DISCUSSION

Urine spots can be stable for long-term storage but must be processed quickly and stored at −70°C. The type of filter paper used also is important. Guthrie cards are optimal for this purpose, particularly because the ash and cellulose content of these cards is known. Smaller aliquots (0.5 to 1 mL) of urine are preferable for assay purposes, but 5-mL aliquots would be adequate for archival storage.

Samples that are frozen without centrifugation pose a particular problem for immunoassays. Some cytokines are impossible to detect in such samples when using commercial kits, although affinity chromatography and mass spectrometry can detect some cytokines.

Urine Albumin as an Example

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Several physiological variables affect albumin levels in urine. Functional proteinuria, not associated with injury, can occur as a result of exercise, change in posture, or fever. Fluid and food intake also can affect urine analyte levels without indicating injury. Albuminuria can reflect glomerular injury to the endothelium, basement membranes, or podocytes, or could indicate tubular injury. Glomeruli normally filter blood, and albumin present in filtrate is then reabsorbed and degraded by the proximal tubules so the final urine normally has an albumin concentration of approximately 10 mg/L, compared to blood albumin concentration of approximately 45,000 mg/L. As the urine traverses the tubules, it is further subjected to denaturing conditions, and changes in pH and ionic strength. Enzymes, peptides, exosomes, and proteins also may enter the urine from the tubule walls during this process.

Collecting and handling can affect the composition of urine. Urine protein level may be influenced by the timing of collection (i.e., first morning void, second void, random voids, timed voids). Differences also occur when urine is collected by a catheter or as part of the initial stream versus midstream. Contamination of urine by microbes, vaginal substances, fecal matter, toilet water, or urine preservatives added to collection tubes also may occur. Data from 127 laboratories indicated that microbial contamination of urine specimens was not uncommon.

Differences in how collected urine is processed also may affect its composition. Formed elements in urine change within 2 hours, and crystals precipitate as specimens cool to room temperature or are refrigerated. Centrifugation at low speed (5 minutes at 400 x g) will separate casts, epithelial cells, red and white blood cells, crystals, parasites, yeast, sperm, and aggregates of Tamm-Horsfall protein (THP). High-speed centrifugation removes bacteria, exosomes, and viruses from the liquid phase. Approximately 10% of total protein is lost if urine is centrifuged at 10,000 x g for 20 minutes. Major urinary proteins, such as albumin, osteopontin, inter-α-inhibitor, prothrombin, and THP adsorb to calcium oxalate and calcium phosphate crystals. Precipitation of crystals from urine occurs on specimen cooling and is affected by pH. Calcium phosphate precipitates at higher pH levels. Uric acid precipitates at lower pH.

Storage affects albumin levels in urine. Proteins can:

- absorb to collection containers;
- precipitate or aggregate;
- be denatured by foaming, freeze-thaw cycles, or variable pH or ionic strength;
- be subject to proteolysis if tubular enzymes, plasma enzymes, white cell enzymes, or microbial enzymes are present; or
- be chemically modified by oxidation, free radical attack, glycation, dimerization or cleavage at pH below 3.

Albumin shows significant variability in stability at −20°C and appears to be more stable at −80°C. However, changes in measured albumin concentration during storage depend on the assay used. Differences in measured albumin are observed when using competitive versus noncompetitive assays or polyclonal versus monoclonal assays. The structure of albumin is relatively well known, and its conformation is known to be affected by ligands and other physical interactions. The structure and heterogeneity of urine albumin can be modified by proteases, chemical modification, reactions of cystein 34 (cysteinylation, dimerization, etc.), and aggregation with other proteins or particulate matter. Western blot analysis of 3-year-old urine samples stored at −80°C showed that the majority of the protein was degraded, but the amount lost differed when evaluated using different assays. The relation of urine albumin structure to its measurement raises questions concerning whether albumin measurement can be standardized. Analysis by high-performance liquid chromatography-time of flight (HPLC-TOF) mass spectrometry shows a high level of microheterogeneity for naked albumin. This microheterogeneity was shown to be sensitive to mass spectrometry preparation methods.

Normalization of albumin for variation in urine volume can be affected by the timing of collection and albumin:creatinine ratios. Urinary creatinine excretion, for example, varies with muscle mass, diet, and creatinine supplementation. Urine is a complex matrix whose components vary broadly. Matrix characteristics such as ionic strength, composition, color, turbidity, presence of albumin ligands, and aggregation of components can affect albumin conformation, and therefore, assays to quantify it. Nonetheless, albumin levels measured by immunoassay versus liquid chromatography-mass spectrometry (LC-MS) showed good correlation in three separate tests. Variability in the measurements may be related to standardization issues.

Albumin has a significant degree of covalent microheterogeneity, and many potential ligands may influence its conformation or structure. The effects of structural variation and the urine matrix may be detected differently in different measurement systems. Each measurement system therefore should be assessed. Additional data on matrix effects and sample stability are needed, as well as studies regarding harmonization of measurement methods.

DISCUSSION

Although albumin structural changes such as dimerization and fragmentation were examined for utility for diagnosis, their usefulness was not validated. New mass spectrometry techniques suggest that some oxidized forms of albumin could be correlated with inflammation in the kidney. Increased levels of glycated albumin are observed in people with diabetes.

Because albumin levels vary over a broad range, assays for its measurements are imperfect and not well-standardized, leading to difficulties in comparing albumin measurements across laboratories. Nonetheless, albumin:creatinine ratios are useful for predicting mortality in large studies. Further work is needed to understand whether different forms of albumin are the cause or indicator of a particular renal problem.

Buffering urine has the potential to improve the consistency of albumin measurements; however, matrix effects and other variables remain problematic laboratory evaluation issues to be overcome.

Sampling Issues that Complicate Other Clinical Urinary Analyte Measurement
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The use of preservatives in urine specimens, preanalytical conditions of the patient, and effects of temperature and long-term storage on urine specimens all have implications for both clinical laboratory service and clinical research using urine. When urine is collected and stored, one must consider the purpose for which the specimen will be used and whether handling conditions can be optimized to allow the matter in question (e.g., clinical biomarker discovery or clinical testing/diagnosis) to be addressed adequately.

Different types of preservatives allow analysis of different types of substances in urine. Preservatives are used to reduce bacterial action, chemical decomposition, or to solubilize and decrease atmospheric oxidation of unstable compounds. Some preservatives interfere with certain analytical methods, which can be problematic when attempting to use either fresh or older specimens. For example, some urinary preservatives act by lowering pH and releasing formaldehyde, and may contain sodium and potassium salts. Therefore, such preservatives cannot be used when urinary sodium or potassium are to be measured. Data are available in standard laboratory texts concerning types of preservatives that can or cannot be used if certain urinary components are to be measured.

The preanalytic conditions of the persons providing urine specimens can affect urine composition and must be documented accurately for both clinical investigation and research. The effects of factors such as age, genetics, ethnicity, and gender can be managed using appropriate reference intervals for each population. It can, however, be difficult to establish appropriate reference intervals, which may vary across ethnic groups. Urine composition also can be affected by diet, exercise, posture, daily and seasonal variations, menstrual cycle and pregnancy, smoking, and alcohol use. Urine levels of analytes such as cortisol, prolactin, aldosterone, renin and iron vary depending on the time of day the urine is collected. Data on diurnal variation, however, are available for only a few such analytes. Additional data also are needed on the effects of temperature and time of exposure to a given temperature on the stability of urine components. The data that are available indicate that storage below $-150^{o}C$ (possibly below $-180^{o}C$) is preferable.

Studies have examined the stability of albumin and other urinary components stored under different temperatures for varying times, with limited data beyond one year. The results vary and their interpretation is further complicated due to the wide variety of analytical methods and conditions encountered in the studies. Measurement of urinary catecholamines has been a classic challenge. In the past, elaborate techniques were

recommended for collection and storage. There were problems with 24 hour urine collections due to incomplete collections, influences of diet and physical activity leading to sympatho-renal activation. This resulted in strong advocacy for spot or overnight collections, though still acknowledging possible confounding in interpretation of results. Currently high-performance liquid chromatography (HPLC) assays are widely used and have improved the quality of routine measurements, with the newer gas chromatography (GC)-mass spectrometry (MS) and liquid chromatography (LC)-tandem mass spectrometry (MS) promising improved analytical specificity. It is now generally accepted that preservation with hydrochloric acid to maintain urine acidity and storage at -80^{o}C to minimize auto-oxidation and deconjugation are optimal. Many drugs, such as antidepressants, vasodilators, alpha and beta-blockers, and dietary stimulants can influence catecholamine levels, however, data on their differential effects in stored samples are lacking.

A rare example of a systematic review of the evidence for collection, analysis, and interpretation of urinary biomarkers was provided by H. W. Vesper et al. relating to the biochemical bone markers urinary pyridinoline (PYD) and deoxypyridinoline (DPD). Concentrations of PYD and DPD could be affected substantially by several preanalytical factors that are easily standardized. It was concluded that urine should be collected at a specific time of day to avoid diurnal variability in the assays for these analytes. Excretion rates from the same type of urine collection should be used for data comparisons. Samples and calibrators should not be exposed to direct sunlight and should be stored at 2^{o} to 6^{o}C if analyzed the same day as collection or frozen at -20^{o}C if analysis will take place more than 24 hours later. Perimenopausal women should not be included in the population used to establish reference intervals. Separate reference intervals should be defined for men, who have higher excretion rates. Because rapid changes occur during childhood, children of the same age or within a 2-year range should be compared, and during puberty, children should be compared to those in the same Tanner stage. Factors that influence creatinine excretion should be recognized and included in data interpretation. Dietary supplement use, particularly vitamin D and calcium, should be assessed, as well as current and previous diseases (hyperthyroidism, hyperparathyroidism, Paget disease, multiple myeloma, fractures, cancers, etc.). Immobilization results in increased excretion of PYD, DPD and creatinine. Immobilization of individuals, therefore, should be considered when assessing results. More systematic reviews of urinary analytes could help to improve their analytical quality and clinical utility.

Some problems relating to storage and stability of analytes in urine have been readily resolved after careful investigation. Significant decreases in uric acid concentration were observed after storage at 4^{o}C and -20^{o}C. High uric acid concentrations and low pH severely affected stability. Diluting the sample 10-fold with distilled water before storage helped maintain stability. Another detailed investigation showed that enzyme immunoassays using monoclonal antibodies were found to be optimal for measuring urinary estrone conjugates. These compounds were stable for 0 to 8 days at room temperature (although significant decreases in estrone conjugates were

seen after more than 2 days at room temperature) and for up to 2 years and 0 to 10 freeze-thaw cycles at −20°C.

Evidence supporting the use of the more convenient spot urine samples compared with the burdensome 24 hour collection is also valuable. A comparison by Ilich et al (2009) of calcium, magnesium, sodium, potassium, zinc, and creatinine concentrations in 24-hour and spot urine samples in women found significant correlations between sodium and calcium, sodium and magnesium, magnesium and calcium, zinc and magnesium, and sodium and potassium in both types of samples. Higher correlation coefficients were generally obtained in spot urine versus 24-hour urine samples. These data may encourage investigators to make greater use of spot urine samples.

An internal review of large epidemiologic studies in which urine was collected found that in most studies only one vial of urine was collected, usually at the beginning of the study. An analysis of methods used for more than 10,000 urine shipments, representing approximately 322,000 urine vials from 70 countries, showed nitrogen vapor was the optimal way to preserve the samples and was less expensive than dry ice, with the average shipment time for both methods being 2 to 3 days.

A urinary analyte measured using different methods may still show consistent clinical interpretation in a study when appropriate reference values for each method are employed. The Heart Outcomes Prevention Evaluation (HOPE) study found that any degree of albuminuria, when measured with a radioimmunoassay procedure, was associated with cardiovascular events and was a risk factor for future events. Reevaluation of the same HOPE samples by HPLC revealed that HPLC detected more people with microalbuminuria than the original radioimmunoassay. Valid and comparable clinical results, however, could be obtained after 5 to 10 years of storage at −70°C and at least two freeze-thaw cycles when method appropriate reference values were used.

DISCUSSION

The general trend in urine preservation is that storage at colder temperatures is better, although handling and thawing also will impact urine stability. At present, there are few studies which address mechanisms of degradation that affect urine analyte stability, such as oxidation or hydrolysis. Albumin also can be degraded by proteolysis. Albumin stability could be affected by a combination of free radical mechanisms and proteolysis. Understanding oxidative degradation may be particularly important if samples are used for metabolomic research.

Urine Handling for Discovery Proteomic Analysis

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Proteins enter the urine by penetrating the glomerular barrier or by being shed from the tubules. Change in glomerular permeability can increase the levels and variety of serum proteins found in urine. Glomerular cells also can be shed into the urine. Changes in renal protein reabsorption and metabolism may occur in some disease states, changing the composition and magnitude of proteins found in urine. Proximal convoluted tubules also contribute peptides to the urine. Exosomes, which are small vesicles excreted along the length of the nephron, reflect the protein composition of that area of the nephron and sometimes are found in urine.

Clinical proteomics involves large-scale, systematic analysis of proteins and peptides (identity, modifications, quantity, and function) to address issues in disease prevention, diagnosis, and treatment. Proteomic analysis involves protein extraction, separation, quantification, and identification. Analysis of complex mixtures of proteins usually involves chromatography and mass spectrometry, although recent advances have been made in chip-based approaches.

Urine handling and storage can affect proteomic results. Freeze-thaw cycles can alter the profile of detected proteins greatly, particularly those in the lower molecular weight fraction. Prolonged storage at higher temperatures (above 4^{o}C) also increases the variation in the low molecular weight peptidome. If a specimen is contaminated by bacteria, bacterial proteins can interfere with proteomic analysis after only 8 hours of growth in the urine specimen.

To begin to address issues pertaining to the impact of urine handling and storage on proteomic analysis, we conducted a study to define short-term freeze-thaw and protease effects on the urinary peptidome and proteome. Normal human urine was frozen at -80^{o}C and subjected to zero, one, or three freeze-thaw cycles in the presence and absence of protease inhibitors. Analysis of the samples at 214 nm showed no difference in the proteomic profiles of the differently treated samples. However, comparison of individual peptide masses that appeared in all samples showed that freeze-thaw enriches some peptides. Some of the detected peptides may be degradation products. Similar effects were seen when peptides were analyzed for the effects of protease inhibitors. The abundance of some species was increased, and that of others was decreased. The cysteines present in some proteins are targets for protease inhibitors, which quench ionization and block detection of the protein. Therefore, the presence of protease inhibitors may decrease the ability to detect cysteine-containing proteins. The effects of protease inhibitors were not highly or broadly significant, but did significantly affect detection of some specific peptides. An analysis of the effects of protease inhibitors found 359 peptides for which abundance did not vary in the presence or absence of protease inhibitors. Of the peptides studied, 137 peptides showed an increase in average abundance in the absence of protease inhibitors, and 64 peptides showed decreased abundance in the presence of inhibitors.

The effects of freeze-thaw cycles and protease inhibitors on some commonly analyzed proteins were examined. Apolipoprotein D showed little change in detection related to freeze-thaw cycles or the presence or absence of protease inhibitors. Albumin showed similarly small effects. This suggests high-abundance proteins are not significantly degraded by freeze-thaw or protease inhibitors. Lithostatine, a low-abundance protein, cannot be detected in the absence of protease inhibitors, and detection of this protein also is hampered by multiple freeze-thaw cycles. Complement decay accelerating factor showed similar effects in the absence of protease inhibitors and when subjected to freeze-thaw cycles. This was consistent with results related to analyses of trefoil, a potential renal injury marker. Gene ontology analysis of the effects of freeze-thaw cycles shows that proteins involved in cellular processes and biological regulation are disproportionately affected by freeze-thaw.

DISCUSSION

To analyze archived samples using proteomic approaches, controls are needed to distinguish true effects from handling artifacts. Different analytical approaches, particularly protein separation techniques, might affect whether freeze-thaw or protease inhibitors influence the measured abundance of protein species. Groups that primarily analyze high abundance proteins would be less likely to detect handling effects. Developing guidelines or principles that predict the effects of handling on particular proteins or peptides will be complicated. Protease inhibitors may be needed to detect low-abundance proteins, but the inhibitors also may impede detection of other peptides and metabolites.

Urinary Handling and Exosome Analyses

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Exosomes are membrane-bound structures found in urinary sediment. They are approximately 30 to 70 nm in diameter and express a specific set of proteins. Aquaporin-2 was identified as an important urinary biomarker for water balance disorders, but because it is an integral membrane protein, its presence in urine initially was puzzling, until it was found in exosomes. Other membrane-bound proteins, such as sodium transporter proteins, also were found to be derived from exosomes and detectable in urine. Type I Bartter patients lack NKCC2, which can be observed by immunoblotting urinary exosomes.

Exosomes are derived from the internal vesicles found within multi-vesicular bodies in the cell. The multi-vesicular body fuses with the cell plasma membrane and releases the exosomes into the extracellular space (urine). Exosomes are excreted by renal tubule cells and podocytes. Proteomic analysis of urinary exosomes has resulted in creation of the Urinary Exosome Protein Database (http://dir.nhlbi.nih.gov/papers/lkem/exosome/). The database contains 1,160 proteins, including proteins known to be expressed specifically in each renal tubule segment and in glomerular podocytes. The database is comprised predominantly of membrane proteins associated with apical plasma membranes as well as

numerous cytosolic proteins. Isolation of exosomes enriches their associated proteins approximately 30-fold relative to whole urine and thus allows detection of proteins involved in renal tubule or podocyte processes.

One barrier to successfully using exosomes to analyze urinary proteins is the presence of THP. This protein is highly abundant in urine and interferes with both two-dimensional gel electrophoresis of urine and analysis by mass spectrometry. Using dithiothreitol in the exosome isolation protocol removes THP so that it does not interfere with analyses. Other barriers to the use of exosomes include the lack of standard protocols for sample collection, storage, and shipping to optimize analysis.

DISCUSSION

Because exosomes are derived from all parts of the kidney, it might be feasible to separate subpopulations of exosomes using antibodies for specific proteins found in certain regions of the nephron. Because it enriches the sample, isolation of exosomes allows analysis of many proteins that probably would not be detectable in urine. Exosomes contain approximately 85% of mRNA found in urine and micro-RNA species as well. Although ultra-centrifugation results in the best yield of exosomes, this is not practical for clinical use. Alternative filtration techniques exist that yield a specimen suitable for immunoassays but not for mass spectrometry.

Urine Handling for Metabolomic Analyses

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Testing for metabolic conditions has been done using urine for more than 30 years, but guidelines for testing were developed only recently. These guidelines include:

- random urine collection (because most samples are collected from pediatric patients),
- storage at -20^{o}C,
- thawing and mixing thoroughly before use,
- use of creatinine as a marker for urine concentration,
- use of internal standards (e.g., isotopically labeled metabolites), and
- extraction of the acidic fraction.

Optimal use of metabolomic analyses for diagnosis and prognostication using urine samples requires determination of optimal preparation and storage conditions and a better understanding of the materials detected using these analyses.

Approximately 200 to 300 individual metabolites can be identified in 0.5 to 3.0 mL urine using gas chromatography (GC)-electron impact mass spectrometry (MS). Liquid chromatography (LC)-time of flight (TOF) MS of 8 μL of plasma yields 4,000 targets (peaks), most of which are not identified. Although analysis of urine GC/MS profiles has shown that these profiles vary in "normal" children, it is possible to use metabolomics to detect certain disorders. For example, 3-methylcrotoyl-CoA carboxylase deficiency generates a distinct GC/MS profile with diagnostic peaks. However, use of

metabolomic profiles for diagnosis may be complicated by excretion of dietary metabolites. A peak attributable to a nutritional additive given to children with neurodegenerative diseases initially complicated diagnosis.

Preanalytical sources of urine metabolomic artifacts include endogenous metabolic sources and exogenous sources, such as drugs (prescribed and over-the-counter) and nutritional supplements. Artifacts related to fasting such as ketones, and artifacts related to stress, such as phenolic acid metabolites of catecholamines also are observed. Symbiotic artifacts also can be observed, such as products of gastrointestinal flora metabolism (e.g., volatile fatty acids, TCA cycle intermediates, and products of bacterial metabolism of plants). Clinical artifacts include metabolites of other disease states such as those detected in patients with liver disease or tumors. Maternal metabolic disease also can cause artifacts that are observable in urine of newborns. Drugs such as anticonvulsants and nonsteroidal anti-inflammatory drugs can create metabolomic artifacts, as can nutritional supplements that are found in formula or product additives to improve palatability (e.g., adipic acid added to make yogurt smooth).

DISCUSSION

At this point, it is unknown whether intact kidney cells present in urine affect metabolomic analyses, but cells generally are not removed from the urine during specimen processing. The presence of kidney cells in urine may affect metabolomic profiles, but this has not been studied extensively.

Urinary Cell mRNA Profiling

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Urinary cell levels of mRNA can be measured using quantitative PCR assays. We used this technique to measure urinary levels of mRNA encoding the cytotoxic proteins granzyme B and perforin, mediators of T cell activity in transplant recipients. This approach provides a noninvasive way to diagnose acute rejection of renal allografts. Acute rejection could be predicted with a sensitivity of 83% and a specificity of 83% using perforin mRNA. Rejection could be predicted with a sensitivity of 79% and specificity of 77% using granzyme B mRNA. Measurement of mRNA for perforin and granzyme B in sequential urine specimens may predict the development of acute rejection. Graft dysfunction attributable to nonimmunological causes also was not associated with an increase in urinary levels of either of these transcripts, nor was bacterial urinary tract infection.

Other molecules that predict the infiltration of vascular cells into an allograft and renal function were examined. An inverse relationship between *FOXP3* (a specific functional factor for regulatory T cells) mRNA levels and serum creatinine was detected during an episode of acute rejection. A linear combination of *FOXP3* mRNA and creatinine predicted rejection reversal (90% sensitivity and 96% specificity) better than *FOXP3* mRNA levels or serum creatinine levels alone. Therefore, FOXP3 provides a

noninvasive way to improve prediction of outcome during acute renal transplant rejection. A clinical trial, NIH CTOT-4: Noninvasive Diagnosis of Renal Allograft Rejection by Urinary Cell mRNA Profiling was developed to test the utility of these findings in the clinic. The trial will enroll 450 kidney transplant recipients to investigate whether levels of mRNA encoding perforin and granzyme B and the T cell marker CD3 are a sensitive and specific noninvasive way to detect acute rejection of renal allografts, and whether acquiring mRNA profiles of sequential urine specimens can predict the development of rejection. Specific urine sample processing protocols were developed involving centrifugation and resuspension of the pellet in a buffer that will preserve mRNA and storage at -80^{o}C. Interim data analysis in 1,927 urine specimens from 394 renal allograft patients showed the transcripts identified in laboratory work were useful for detecting rejection. Urinary cell mRNA levels for CD3, performin, and granzyme B predicted acute rejection with a sensitivity of 95% and specificity of 79%.

DISCUSSION

Approximately 10 to 15% of the samples had insufficient levels of mRNA for analysis. The urine was qualified by determining cell numbers. No restrictions were placed on how the urine was collected (e.g., time, midstream, and etc.) because to be practical in the clinic, the assay should not be affected greatly by these conditions. In most cases, the differences in mRNA copy number were large enough that specific collection procedures were not needed.

Sample Collection Challenges for Clinical Care: Collection, Transport, and Storage
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Regulatory agencies have asked that clinical laboratories maintain control over pre-analytical, analytical, and post-analytical processes. Pre-analytical processes include physician discussions and decisions to use a particular test; clear requisition processes; instructions to patients; use of appropriate collection materials and containers; appropriate handling, processing, and storing of the sample; appropriate transportation conditions; and sample delivery to the laboratory.

Analytical processes include receipt and log-in of samples by the laboratory; appropriate routing within the laboratory; maintenance of specimen condition; testing within the stability period of the sample; appropriate treatment of the sample to avoid sample loss or analyte degradation; full validation of tests, and qualification of personnel; appropriate instrument and reagent maintenance; thorough assessment of results; and release by a qualified person. Post-analytical processes cover delivery of results to the client; correct calculations and data delivery; correct conversion to SI; and data storage.

When collecting urine specimens for testing, physicians must be aware of how the urine will be used to ensure that it is collected and preserved in an appropriate manner. Questions to be answered pertaining to sample collection include whether the physician will supply collection items; whether preservation is required; the amount of urine to

collect; whether urine will be collected once at random, overnight, as a 24-hour or 12-hour specimen, or in another manner; whether the patient will need to store the urine and how he or she should do so; and whether dietary restrictions or dietary monitoring are associated with the test.

Transportation considerations include whether special supplies or packaging are needed, whether aliquoting must be performed at a regional laboratory, the temperature required during transport, any special receipt requirements at the laboratory, and whether the specifications are driven by stability data pertinent to the analyte or test. Storage of samples in a commercial laboratory is determined by whether additional tests or repeat of the initial test on a given sample are anticipated, the expected services provided by the laboratory and any relevant regulations. Samples typically are stored only for a short time at commercial laboratories unless otherwise specified. Customers may require specialized storage (e.g., ambient, refrigerated, frozen or vapor phase) and also may require validation and monitoring of freezer storage to ensure maintenance of the desired temperature. For pharmaceutical clinical trials, documentation of correct sample storage and handling often must be provided.

The CLSI seeks to improve the quality of medical care by developing best practices in clinical and laboratory testing and promoting use of these practices worldwide. CLSI uses a consensus-driven approach involving industry, government, and health care professional viewpoints to develop and disseminate these practices. CLSI has developed documents on methods evaluations, including analysis of body fluids, use of mass spectrometry in the clinical laboratory, reference intervals, and evaluation of matrix effects.

Sample Collection Challenges for Clinical Research: The Cooperative, Multicenter Trial, the Research Laboratory, and the Repository
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Large-scale epidemiologic studies require collection of samples at field centers and often require local storage and shipping. The most common measurements on urine performed in multicenter epidemiology studies are for creatinine and albumin. Other clinical chemistry analytes such as total protein and sodium are measured, as are some specialty biomarkers such F2-isoprostanes (oxidative stress) and cotinine (smoking). Measurement of some of these less commonly used biomarkers has not been highly successful in large-scale settings. Multicenter studies and trials will continue to use clinical laboratory measures to help define the outcomes of interest, but as exploration of the underlying physiology and pathophysiology grows, both “standard” collection protocols and “specialty” collection protocols will be needed.

Different types of collection procedures are used in multicenter studies, including 24-hour timed collections (for analytes with diurnal variation), timed collections (e.g., time of day or after a meal), first morning collections, and random collections. First morning collection and random collection are commonly used in population-based studies, and urinary creatinine is usually ascertained to help standardize measures.

Although most clinical laboratory guidelines recommend testing urine within 2 hours of collection, this often is not feasible for multicenter studies. Most samples are refrigerated or frozen. In some cases, vacutainer-type systems have been developed for collection/transportation of specimens, which have the capacity to include appropriate preservatives for specific types of urine analyses. As new biomarkers and analytes come into use, special additives likely will be required.

Because of size and logistical issues, multicenter studies often must make compromises in collection and handling protocols. Field centers often are underfunded and understaffed, lack important pieces of equipment, face sudden changes (such as participants who cancel or staff absences), and experience wide variations in participant accrual. Because of these inconsistencies, questionnaires may be incomplete, fewer biospecimens (fewer time points or fewer tube types) may be collected, fewer patients may be accrued, and data on fewer variables are collected. Specifically for urine collection, random sample spot collection may occur rather than timed collection, the collection quality may be compromised (partial collection and contamination versus midstream clean catch), refrigeration may or may not be possible, and processing could be inconsistent or delayed versus performed quickly and according to a strict protocol. Multicenter studies involved in biospecimen handling must consider whether samples can be refrigerated immediately, whether special preservatives are required (and in what form), whether special handling is required (centrifugation, filtering), how samples will be frozen, what size aliquots will be kept, and what sorts of tubes or vials and labeling procedures will be used.

Specimens from multicenter trials usually are stored in a central repository. Repositories must decide on the number and volume of aliquots that will be saved, considering handling and freezer space costs. The size of the aliquot stored often depends on the types of assays that will be performed. Barcoding of specimens has become a necessity.

Repositories also must ensure that Material Transfer Agreements (MTAs), NIH Third Party Agreements, and ethical, legal, and social issues (ELSI) and Institutional Review Board (IRB) consent issues are addressed. These agreements need to anticipate post-study disposition of residual biospecimens, and eventual transfer to sponsoring organization biorepositories (such as the NIDDK Biorepository). Special challenges include the long-term coordination of clinical information with the biospecimens, as well as consideration of the terms of informed consent agreements which outline conditions regarding how the specimen can be used. Not uncommonly novel technologies, not anticipated at the time of collection, can be applied to residual specimens, depending on the original consents.

Several large multicenter studies have developed protocols for specimen collection, including protocols for preparation of participants, collection schemes, and urine processing (including aliquoting and use of preservatives or other additives). Unfortunately these protocols may be focused on the disease process of interest and unintentionally limit the capacity of specimens to be shared for future studies, or

validation of biomarkers. It is unlikely that development of a universal collection scheme for large trials is possible, particularly for future, currently undefined assays and analyses. However, a feasible goal is the development of minimum standardized collection schemes that address certain classes of assays (e.g., immunoassays or assays for oxidative stress analytes). Developing a standardized collection approach may facilitate comparison of results across trials.

DISCUSSION

Although it is unlikely that a standard urine collection scheme that can be used without modification across numerous types of trials could be developed, clear instructions for urine collection should be developed. Reproducible artifacts that are detected in specimens could be one way to "standardize" an assay. Human error could be addressed by using blind duplicates; however, most errors are made in collection rather than processing. Filtering and lyophilizing specimens has worked well for many assays and may be one way to circumvent space issues when storing large numbers of urine specimens.

Urine Biospecimen Handling: Lessons from NHANES

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The National Health and Nutrition Examination Survey (NHANES) is a cross-sectional survey of the U.S. civilian, non-institutionalized population. Health information from survey participants is collected by home interview and physical examinations (including sample collection) in the NHANES Mobile Exam Center. Over the years, NHANES has learned lessons that have helped ensure the success of sample collection. The NHANES 2009-2010 examination included collection of urine specimens. The response rate for urine collections taken between 1999 and 2009 was 96%, with only 4 % of those samples having insufficient quantity. 65 mL of urine is collected from children between the ages of 6 and 11 years, and 75 mL is collected from teens and adults. In the Mobile Exam Center laboratory, the urine is aliquoted into 14 vials, barcoded with participant ID and laboratory test code, frozen at -20^{o}C, and shipped to laboratories on dry ice weekly. For successful urine processing and handling, NHANES has learned that it is best to process only one urine specimen at a time, and to test container labels in advance to ensure that they can withstand freezing and thawing. NHANES tests for several substances in urine, including albumin, creatinine, caffeine, heavy metals, pesticides, phytoestrogens, and organophosphates. Testing urine containers in advance to determine that they are not contaminated with the substance being studied also has been found to be necessary. NHANES asked participants to collect a second urine sample at home within 10 days of the initial examination.

Participants were asked to collect the first morning void and to send the urine to the laboratory. Of those who agreed to take the kit home (95% of participants), 88% successfully mailed a specimen to the laboratory.

NHANES established a specimen bank in 1991 and began banking 5 mL of urine in 1999. The specimen bank currently contains more than 2,000,000 urine, serum, and plasma specimens. NHANES uses separate consent forms for the interview, examination, and for permission to store specimens. The consent to store specimens was separated from the examination consent so that refusal of sample storage did not jeopardize participation in the health examination. When genetic research was included in the specimen storage consent form, 85% of participants consented to having their sample stored. In contrast, 98% consented when the forms indicated that specimens would be used for non-genetic research. For minors, permission from parents or guardians was documented, as was assent to participate from participants between the ages of 7 and 17 years.

NHANES is considering whether or not minors should be recontacted when they reach the age of majority to obtain consent for storing their specimens. NHANES consent forms indicated that no specific studies were planned, and future research proposals would be reviewed for scientific merit and ethical considerations. NHANES made the decision not to contact individuals with individual test results. Clinically relevant tests, therefore, cannot be performed on the specimens. Participants are assured of confidentiality and can remove their samples from the repository at any time.

Investigators may submit proposals to use NHANES specimens. Proposal guidelines are available on the NHANES website. The proposals are subject to technical as well as IRB review. Samples are released only after a study is approved, at a cost of $8.50 per vial. At this point, NHANES has not performed methodological studies to determine the effect of shipping or freeze-thaw cycles on banked urine analytes, and also has not performed longitudinal assessments of untouched NHANES urine samples to determine the effects of long-term storage.

DISCUSSION

The tests that NHANES performs on urine samples are based on investigator research interests, as determined by the proposals received by NHANES. Most IRBs have determined that if minors cannot be re-consented once they reach 18 years of age, their data must be removed and samples destroyed. However, the Office of Human Research Protection (OHRP) has stated that IRBs should consider whether obtaining a waiver of consent would be possible. Because of its decision not to provide results to participants, NHANES has not yet allowed proposals that test for clinically relevant information.

Viewpoints From the FDA -- The Biomarker Qualification Process
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FDA's Genomics Group reviews Voluntary Exploratory Data Submissions (VXDS), reviews data submitted for New Drug Applications (NDA)/Biologic Applications (BLA)/ Investigational New Drug Applications (IND), and formally regulates the biomarker qualification process. Given the large increase in submissions of potential genomic biomarkers, the Genomics Group also plays an important role in outreach and communication, education and training, and policy analysis related to genomic biomarker development. The Genomics Group conducts VXDS meetings, at which sponsors can submit exploratory biomarker data for review by FDA staff to receive feedback about additional data needs required for qualification of the biomarker.

The FDA definition of "qualification" for a biomarker covers several areas and refers to the application or use of the biomarker to make a decision, particularly a regulatory decision. The biomarker must have been demonstrated to reliably support a specified manner of interpretation and application within a carefully and specifically defined "context of use." The biomarker must show utility in drug development, which is central to the purpose of qualification. Qualification cannot be granted if there are serious study flaws in collecting data, attempts to apply the biomarker outside the qualified context of use, or if new scientific evidence conflicts with prior conclusions. Context of use is the key to qualification and will inform biospecimen handling for use of the biomarker. Context of use includes the manner and purpose of use for the biomarker (e.g., a range of clinical disorders, drug classes, or species), includes procedures and criteria for obtaining samples and interpreting results, and defines the boundaries of known reliability. Certain biomarkers may have value outside of a demonstrated context of use. These are considered on a case-by-case basis and require further work to expand the qualified context of use. The FDA Biomarker Qualification Process consists of a consultation and advice stage that includes informal discussions with a potential biomarker sponsor and evaluation of submitted data, and a review stage, for which the Biomarker Qualification Review Team (BQRT) receives the full data package and writes the draft biomarker qualification review. The submitted biomarker and its data are presented at a Center for Drug Evaluation and Research (CDER) Regulatory Briefing, and the CDER Office Directors decide whether to accept or reject the recommendations made by the BQRT.

FDA has received at least 23 proposed exploratory biomarkers of kidney toxicity. Seven have been prioritized for FDA/European Agency for the Evaluation of Medicinal Products (EMEA) submission: urinary albumin, β2-microglobulin, clusterin, cystatin C, Kim-1, TFF3, and Total Urinary Protein. Nonclinical qualification of Kim-1 and albumin compared these biomarkers to BUN and serum creatinine levels in the context of a histopathology "gold standard" of kidney damage observed in a rat model. This analysis found that albumin could better detect kidney damage than BUN and serum creatinine, and that Kim-1 was better than all three at detecting low levels of damage. FDA and

EMEA concluded that these renal biomarkers were acceptable in the context of nonclinical drug development for the detection of acute drug-induced renal toxicity. These biomarkers provided additional and complementary information to currently available standards. Kim-1 and albumin could potentially be used in the clinic to determine reversibility of histopathology.

Plans are under way to assess the utility of urinary Kim-1 and albumin in Phase I/II clinical trial decision-making and for diagnosing otherwise non-monitorable toxicity.

Issues that remain to be addressed include how to determine the utility of the biomarker in the absence of gold standards and how to establish thresholds against both healthy volunteers and newly discovered subphenotypes. Ethical and legal issues may arise related to samples and their distribution, but the Genomics Group has found that VXDS analyses can be conducted accurately using fully anonymized data. Investigators nonetheless must consider how informed consent should be structured to reflect future analytical use of samples, particularly genetic analyses. Policies that are clearly defined, flexible, and can be modified as needed over time must be established to govern access to samples. New analytical tools may be needed to evaluate biomarkers, particularly in cases for which a "gold standard" may not be available, but this is less problematic than constraints on the size of patient populations in qualification studies and weak phenotypic definitions for the identification of major biomarker effects.

DISCUSSION

The most significant challenge faced by investigators wishing to qualify biomarkers is the lack of samples with adequate phenotypic information to allow proof of association between the biomarker and the condition it is meant to detect. Incomplete phenotypic information is a problem in many large studies.

FDA has not yet developed strict guidelines for urine biomarkers because data needs may change depending on the biomarker. Approximately 10 genetic biomarkers (detectable in plasma and urine) are in the process of being validated, including two sets of kidney urine biomarkers. A submission recently was received for a proteomic biomarker of chronic kidney damage that is assayed using electrophoresis and mass spectrometry. Data for this biomarker were generated using frozen samples.

Material Transfer Agreements

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A Material Transfer Agreement (MTA) is a contract governing the transfer of tangible research materials or data between two organizations when the recipient intends to use the materials for research purposes. MTAs are contractually binding and must be created with care. In the case of a disagreement, the courts may parse the words of the contract to determine assignment of intellectual property rights. The Bayh-Dole Act of 1980 allows federally funded researchers to retain title to an invention if they file a patent application, attempt to commercialize the invention, share royalties with the inventors,

and grant a non-exclusive license to the government. The Bayh-Dole Act was intended to motivate commercialization of invention and has led to the creation of more than 2,000 new biotechnology companies and more than 250,000 jobs. “Invention” is a legal concept referring to the claimed subject matter. The “inventor” is the person who conceives the ideas, and not necessarily the person who puts the idea into practice or use.

The NIH Research Tools Policy states that sources and suppliers of biospecimens have no inherent rights to inventions made using biospecimens. Inventors, however, may enter contractual obligations to suppliers through MTAs. Three types of MTAs exist: (1) between academic (nonprofit) institutions; (2) from academia to industry; and (3) from industry to academia. Agreements between academic institutions can be governed by the Uniform Biological Material Transfer Agreement (UBMTA), which simplifies negotiations by providing standard contract terms and definitions. UBMTAs generally are not used between academic and industry entities, in part because investigators may have various obligations under an industrial MTA that do not comply with the UBMTA.

Issues that may require negotiation when creating an MTA include confidentiality, delay in publication, definition of “material,” control of intellectual property, and conflicts with existing agreements. Industry may require nondisclosure of confidential information to protect itself from competitors. This may conflict with academic investigators’ desire to publish results. To address this, companies may require a review period for publications. Premature publication may cause loss of patent rights, as may public remarks (such as those made at scientific meetings). Public disclosure could create problems with patent protection, but a patent application must provide an enabling disclosure, that is, a statement noting that a skilled user can use the invention without “undue experimentation.” The definition of “material” must be stated clearly in the MTA, because “material” may include investigators’ modifications or derivatives, and an inaccurate definition of “material” could cause the investigators to lose ownership of the modifications they have made. Control of intellectual property may restrict investigators in their ability to interact with other parties, such as extending licenses. MTAs also must address conflicts with existing agreements, such as obligations to the research sponsor or material provider, or conflicts with the Bayh-Dole Act.

An example of conflicts in ownership of an invention is the case of Stanford v. Roche, in which rights for PCR assays for monitoring HIV RNA as a marker for AIDS drug efficacy were contested. Stanford inventors worked with Cetus, which provided PCR expertise and reagents, and had contractual obligations assigned to Stanford and Cetus. These obligations created conflicts between the assignment of rights to Cetus (which was acquired by Roche) and the provisions of the Bayh-Dole Act allowing Stanford to retain title independently of assignments to a third party. Roche contended that Cetus provided the expertise and techniques that led to the patent and should be recognized just as holder of the intellectual property rights to the invention. Unclear wording in the MTA raised doubts about the assignment of rights to the invention. Roche has asked for a rehearing on the matter.

Ethical Considerations in Assessment of Urine Samples

Marianna J. Bledsoe, M.A., Deputy Associate Director, Clinical Research Policy Analysis and Coordination Program, Office of Biotechnology Activities, Office of the Director, NIH

Ethical and regulatory issues for specimen collection, distribution, and use include participation of human subjects, privacy issues, informed consent requirements, and IRB review. Regulations that may apply to the collection of human specimens include the "Common Rule" (45 CFR 46), which governs research with human subjects conducted or supported by federal departments and agencies, FDA human subjects regulations, Health Insurance Portability and Accountability Act (HIPAA) privacy and security rules, the Privacy Act of 1974, state laws, international regulations or guidelines, and the Genetic Information Non-Discrimination Act (GINA). Use of specimens constitutes human subjects research under the Common Rule if the specimens are collected through intervention or interactions with an individual, a research repository or database is created by gathering identifiable private information or specimens, or if identifiable private information or specimens are obtained from a research repository or database. The OHRP has ruled that creation of a repository is classified as a research activity. The Common Rule provides guidance on situations in which use of data or specimens does not constitute human subjects research.

FDA regulations regarding human subjects research differ slightly from 45CFR 46. FDA regulations state that use of a specimen could constitute human subjects research even if the sample has been de-identified. Situations in which humans participate in research either as recipients of a test or as control participants, or in which an investigational device is used on individuals or their specimens also are considered human subjects research. IRBs may waive consent under 45 CFR 46 for minimal-risk research if certain conditions are met. However, FDA exemptions for informed consent are limited to emergencies, life-threatening situations, or military operations. FDA has stated that it will exercise enforcement discretion regarding consent under certain circumstances in which human specimens are used in FDA-regulated *in vitro* diagnostic device investigations.

An informed consent form for prospective specimen collection must meet the requirements of human subjects regulations. It also must include descriptions of the specimens/data and process used for collection, risks to privacy and confidentiality and methods to mitigate risk, the purpose of the collection and conditions for sharing, and the types of research that will be conducted. Informed consent forms also should contain statements concerning the right to withdraw from the study, whether results will be returned, and any plans for recontact.

When appropriate, information on the consequences of DNA typing, details regarding specimen storage, and fate of specimens/data when no longer useful, when the project loses support, or when the collection is transferred to others should be provided. Informed consent forms should be clear and understandable and should avoid restrictions

that may prohibit future use of specimens. In some cases, it may be appropriate to use "tiered consents" to allow volunteers to participate in only some aspects of a study and to provide choices regarding how specimens will be used.

Investigators also should be aware that HIPAA authorization, or waiver of authorization, may be required depending on the type of data collected and whether the data are being collected or used by or on behalf of a Covered Entity (e.g., a health care provider that conducts certain transactions in electronic form, a health care clearinghouse, or a health plan). For secondary use of specimens, the content of any existing consent should be considered even if only de-identified or anonymous specimens are used. Consensus is developing that, from an ethical perspective, secondary uses should be consistent with the initial consent under which the specimens were collected. This challenge is not infrequently encountered. An example might be the collection of biospecimens during a clinical trial for measurements of proteins, coupled with a subsequent proposal to apply nucleic acid based assays to the residual specimens after the trial has been completed. These "additional use" applications must be appropriately covered by the initial consent for use. The HIPAA Privacy Rule may apply, depending on the information associated with the specimens.

Well-documented policies should be developed for specimen sharing and access, including procedures for determining appropriate research uses. These polices may be based on both scientific and ethical considerations. Identifying information should be removed whenever possible, in accordance with applicable regulations. IRB review should be documented, and confidentiality agreements and investigator usage agreements/MTAs should clearly define the rights, obligations, and responsibilities of the specimen provider and the recipient.

Ownership issues are of particular importance for specimen collections. Unresolved ownership issues have been at the root of three recent court cases. In Moore versus Regents of the University of California, the court ruled that John Moore had no ownership claims to new products developed through use of his specimens. Researchers, however, were advised to reveal their financial interests to specimen donors. In Greenberg et al. versus Miami Children's Hospital, families of children with Canavan disease created a biorepository and required investigators to perform research on the disease using the specimens. The families sued when a commercial test was developed whose use was licensed at a high fee, claiming that they were not told that commercial tests would be developed. The court ruled against the families. In Catalona versus Washington University, an investigator built a large collection of prostate cancer specimens and tried to bring the specimens with him to a new job, but Washington University claimed ownership of the specimens. Catalona asked the donors of the specimens to withdraw their consent and transfer the specimens to him, but the court ruled in favor of Washington University. In all three cases, individual ownership of excised tissue was not recognized. These cases underscore the importance of defining custodianship, which differs from ownership. Custodianship implies caretaking responsibility, including governance, management, and oversight; conditions for access and use; and documented plans for disposition of specimens and data if a project loses

funding or a repository closes and plans for transfer if the custodial investigator leaves the institution.

Issues may arise when specimens are collected from members of special populations. Certain populations have specific beliefs regarding the disposition/handling of specimens, their storage, and/or export. Export laws may apply if specimens are transferred internationally. Community consultation/involvement regarding specimen collection and use may help mitigate some of these issues in certain situations. Investigators also should consider potential group harms from specimen research. For example, use of biospecimens from Havasupai tribe members in migration studies challenged tribe members' beliefs about the origins of their group. This example demonstrates issues that can arise even when anonymous specimens are used. Return of research results also can raise issues for investigators. Conflict may arise between the rights of individuals to information about themselves versus harms associated with inappropriate return of individual research results. For example, harm may occur if individual results that have not been analytically and clinically validated, or are of unknown clinical significance, are returned to an individual who provided a specimen. Clinical Laboratory Improvement Amendments (CLIA) 1988 prohibit the return of results for clinical care if the tests are not performed in a CLIA-approved laboratory. Investigators should establish mechanisms for making decisions about when to return clinically significant individual findings. Informed consent forms should address if and under what circumstances subjects will receive individual results. In some cases, it may be appropriate to provide generalized, aggregated research findings to subjects.

The International Society for Biological and Environmental Repositories has developed a document on best practices for repositories, titled "Collection, Storage, Retrieval, and Distribution of Biological Materials for Research." NCI also has defined best practices for biospecimen resources. These are described at http://biospecimens.cancer.gov. HHS and NIH activities addressing ethical issues associated with specimen research include the Clinical Research Policy Analysis and Coordination Program, the Secretary's Advisory Committee on Human Research Protections, and efforts in other individual NIH institutes.

HIGH PRIORITY SUGGESTIONS FROM BREAKOUT GROUPS

Group 1: Collection, Handling, and Long-Term Storage

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Preface

The recommendations on urine collection, handling, preservation of biomolecules and storage are based primarily on experience and expert opinion. There is limited published evidence in support of many of the recommendations. The recommendations for urine handling and analyte stability in general textbooks are limited to the intended use for diagnosis and management of patients, and contain limited and sometimes conflicting information on long-term storage. The recommendations in this report should be considered as subject to change as data become available. A section on research recommendations suggests areas where additional evidence is needed.

Urine sample collection

Urine samples should be collected in a manner to avoid contamination from tissues near the urethral meatus and from cleansing agents. The urine will have already had an unknown incubation time in the bladder prior to urination that may contribute to alteration of biomarkers (for example, action of proteases). Consequently, recording the time of the prior urination, when available, is important. Urine samples should be transported promptly to the laboratory where testing or storage will take place (see later section). Procedures are the same whether collecting urine from inpatient or outpatient settings.

Collecting urine through a catheter implies additional considerations. Catheter placement may cause inflammation products and blood to be in the urine, which is unrelated to a pathophysiologic condition. Catheters are more likely to be used when a patient has unstable kidney function or acute kidney injury, consequently normalization of biomarker concentrations to creatinine may be inaccurate. A critical issue that needs further investigation is the influence of binding of molecules to the catheter surface and differences in binding affinity to different catheter materials. It is important to document catheter use, type and material of catheter construction, duration of catheter placement, and date/time of urine collection.

It is difficult to recommend a specific time of day for collecting a urine sample. The first morning void is influenced by a longer time in the bladder but is usually the most concentrated sample. A first or second morning void is preferred to a random void urine sample because the intra-individual variability in albumin concentrations is lower (1). Random samples are frequently used for pragmatic reasons. In general, a 24 hour sample should not be used because performing a complete 24 hour collection is difficult and changes during storage may influence biomarkers. Collection of a 24 hour urine sample needs to be justified by specific study goals, for example: balance studies, to determine excretion rate (in both cases, consider if a shorter time interval is suitable), or for patients with acutely changing kidney function. Single void collections can be normalized to urine creatinine to adjust for dilution effects except in conditions when creatinine is a poor marker for GFR, such as diseases affecting rate of generation related to muscle mass and diet or the rate of tubular secretion, and in patients with rapidly changing kidney function.

Urine Sample Processing

Urine processing should be performed by staff knowledgeable in laboratory procedures who are specifically tasked and trained in the protocols required for preservation of biomolecules. Hands-on training is essential and staff should have regular (minimum annual) competency assessment in correct procedures (2,3). The principal investigator and/or a trusted assistant should conduct spot-checks to ensure that standard operating procedures (SOPs) are correctly followed. Research urine handling protocols should mimic as much as possible typical clinical use protocols to minimize errors and to speed translation of research findings to clinical practice.

Urine contains particulate components and many molecules that may precipitate when cooled to refrigerator temperatures. Consequently, it is recommended to maintain freshly collected urine at 20-40 °C and centrifuge within 2 hours of collection. If rapid centrifugation is not possible, the urine should be kept at refrigerator temperature (2-4 °C), allowed to warm to room temperature and be thoroughly mixed before centrifugation (see later section for conditions). If there will be more than a 24 hour delay in processing, the urine should be frozen, then allowed to thaw to room temperature and be thoroughly mixed before centrifugation.

It is recommended to characterize the freshly collected urine using a multi-parameter dipstick test (in a separate aliquot that is discarded) because this information is not reliable when performed on a stored sample and is valuable to characterize the urine at the time of collection. When feasible, it is suggested to perform a urine creatinine measurement on the initially collected urine sample to assist with selecting stored samples for future testing. Also, consider if measuring specific gravity using a refractometer is appropriate for a study's objectives. It is also recommended to collect serum and dipotassium EDTA plasma samples paired with a urine sample in order to assess the fluid filtered in any subsequent biomarker investigations.

The urine collection and processing SOP must be detailed and as standardized as possible among different research investigations. Detailed instructions are required for patient procedures, urine collection, handling, transfer, and processing steps. It is generally prudent to process and freeze samples within two hours or as quickly as possible to preserve biomolecules for future investigation. The procedures for thawing and mixing frozen samples must be provided. Dissolved and sedimented molecules will concentrate and layer differently in urine with different pH, concentration of solutes, freezing rates, thawing rates and mixing procedures. Written procedures must contain very specific details about how to perform all sample handling steps to ensure reproducibility within and ideally among investigations. Particular molecules known to be of interest before the start of sample collection should be investigated for stability throughout the proposed handling SOPs to ensure best possible outcomes for the desired target biomolecules.

Labeling and sample tracking errors should be minimized by using bar-code labels and appropriate electronic data entry systems. All tubes and containers should be labeled prior to sample collection and processing. Manual processing steps which introduce the possibility of mislabeling samples, such as transferring urine between containers and aliquoting, should always be performed one sample at a time or should be automated.

Transcription errors are unavoidable when recording data associated with urine collection or measurements. An approach to minimize transcription errors is to use a double data entry system in which two different people enter the same numerical data into an electronic file. An automated process can be set up to reconcile entries, identify discrepancies, and allow correction by review of the original source data.

Urine samples for long term storage

The priorities for preparing urine samples for storage are: 1) to meet the planned needs of the specific study, and 2) to support the future unanticipated needs of the parent study or other future research. For the latter, long-term storage of samples in a biorepository is recommended. Some funding agencies now require samples to be deposited in a biorepository. Biorepositories offer a high level of quality assurance and sample control which is difficult for individual investigators to achieve. Although the preparation and storage of multiple aliquots entails added up-front costs, these costs will

very likely be offset by savings when additional measurements are needed for a specific study and suitable samples are available. The aggregate research experience is that unanticipated biomolecule measurements are frequently needed, but cannot be made because suitable aliquots were either not stored or were stored under inadequate or unspecified conditions that compromised the validity of the measurements. Saving the proper number of aliquots under suitable storage conditions (see below) and avoiding freeze-thaw events contribute to cost avoidance by eliminating, in many cases, the need for additional subject recruitment. It is recommended that investigators involve a representative of a biorepository in the planning process of all clinical trials needing long-term storage of samples in order to ensure that sample processing, labeling and storage are compatible with the biorepository requirements.

It is recommended to prepare and store the number and type of aliquots shown in Table 1 to support follow up and future testing on the samples.

Table 1. Urine aliquots to be stored in a biorepository for future use.

Minimum aliquots	PELLET	CLEARED	ACID (HCI)
Desirable aliquots	Insoluble material	Protease inhibitor	Acetic
	Cytology	Buffer	Boric
	Endosome	Diluted	
	Exosome	Alkalinized	

The minimum three aliquots that will permit a large range of future testing to be performed are: a low speed centrifugation pellet, the cleared supernatant after centrifugation, and an additional aliquot of the pre-centrifugation urine acidified (with HCl to pH 2) to dissolve any precipitated compounds. A portion of intact urine sample should be centrifuged at 400 x g for 10 minutes to obtain a pellet that will contain minimally altered cellular and cast components, and insoluble material. The pellet can be subdivided and a portion preserved in an RNase inhibitor for RNA analyses (4), and with other preservatives as needed for other specific components. It is also desirable to centrifuge an aliquot of the urine at higher centrifugal force (e.g. 2-3000 x g) to more completely clear the urine of very small insoluble precipitates (which should also be saved) that could adsorb other biomolecules and alter the recoverable concentrations.

Several additional aliquots are desirable to have available a range of urine samples using various approaches to stabilizing compounds. Aliquots preserved with acetic and boric acid will provide a different pH to optimally stabilize some biomolecules (5). Addition of protease inhibitors (6,7,8), buffers to control pH, diluents to reduce the concentration of inorganics to avoid precipitation, and alkalinization to stabilize some types of molecules (7,9) will create an array of sample types that will enable recovery of biomolecules with a range of sensitivities to degradative processes. The centrifugation conditions and preservation of the pellet should address requirements to stabilize the cellular elements for cytologic examination and to preserve exosome and endosome structures. For example, protease inhibitors are required to preserve proteins found in exosomes. In addition, vigorous vortexing of urine samples is required to recover

exosomes from frozen urine (10). There was not agreement regarding which protease inhibitors would be most suitable, and there was concern that some protease inhibitors may bind irreversibly to the target proteins, which could complicate future mass spectrometry analyses. Urine sample preparation issues for Tamm-Horsfall protein analysis have been described (11,12).

For each aliquot condition, a minimum of four 0.5 mL samples should be saved in 0.6 mL total volume cryovials with silicon gaskets, plus a 15 mL volume in a larger cryovial. In all cases, aliquots to be frozen must occupy 50-90% of the total volume capacity of the container. The minimum 0.5 mL and 50-90% volume fraction are important to avoid losses due to freeze-drying effects when the liquid surface to air ratio is excessive. The recommended container to store frozen pelleted materials needs further investigation. The labels used on the containers need to be compatible with the freezing procedure and storage temperature. A bar code or other computer readable label is required to minimize the time spent locating samples which will minimize any warming that may occur during handling in the frozen state.

The colder the temperature for long-term storage of urine biospecimens, the more likely it is that biomolecules will be stable. Various thermal transitions occur in frozen water at different temperatures and are influenced by the concentrations and solutes in the water. Faster rates of freezing are preferred to minimize the time spent in various transition states. Urine is a complex aqueous fluid with a wide range of matrix components that can differ markedly with the physiologic condition of individuals. The solutes are excluded from the crystal lattice of water as freezing occurs and the hydration properties of solutes will change. Consequently, frozen urine will not have a homogeneous distribution of solute molecules and the temperature at which water molecules are fully immobilized (i.e. loss of molecular motion, diffusion and chemical reactions) will be different for different urine samples but is not likely to occur until at least -70 °C and may be lower.

The temperature for short-term storage is -70 °C or lower. Long term storage at -70 °C or colder has been the minimum standard of practice, but less than -130 °C, the glass transition temperature for water at which chemical activity of water ceases, in the absence of solutes is recommended for new projects. Storage temperatures below -130 °C can be attained using liquid or vapor phase nitrogen freezers or mechanical cryofreezers. Storage in liquid nitrogen has been associated with sample contamination due to entry of liquid nitrogen into cryovials (13,14). Storage in vapor phase nitrogen freezers reduces the risk of contamination, but considerable temperature gradients have been reported within vapor phase freezers such that samples can reside above the glass transition temperature depending on their location within the freezer (15,16). Mechanical freezers provide uniform temperatures throughout the storage compartment, but have higher operating costs, require relocation of contents for defrosting, and are prone to electrical or mechanical failure. Transportation of frozen samples can be accomplished with solid carbon dioxide (dry ice), or using shippers with vapor phase of liquid nitrogen. Storage and shipping using vapor phase may be less expensive than the mechanical

refrigeration used for -70 °C systems or shipping with solid carbon dioxide, and shipping delays are less likely than when solid carbon dioxide is used.

Regardless of the storage temperature it is important to avoid thermal stress when handling apparently frozen samples. Defrosting freezers or searching for samples in a freezer can cause 20 °C or more warming with attendant increases in molecular motion and risk for chemical degradation reactions to occur. It is recommended to carefully manage thaw/freeze cycles to maximize testing performed when a sample is thawed. Finally, -20 °C must not be used for storage of urine samples because, although some molecules may be stable for short time periods at -20 °C, many will not be stable and the intent is to preserve samples for future examination. Frost-free freezers must not be used because these freezers maintain a frost-free condition by continually undergoing thaw/freeze cycles to vaporize frost films.

Documentation

It is necessary to maintain detailed documentation of the urine collection, transportation, processing and storage conditions. This information is needed to conform to requirements for a specific investigation, but is also needed for any future use of stored samples. When considering biorepository requirements it is essential to document all changes (or lack of change) in storage conditions, including relocation of samples for defrosting, temperature changes due to refrigerator failures, and any thaw/refreeze cycles a sample may have encountered. Continuous temperature monitoring records are required and ideally will include devices located in subcompartments of a storage system. The documentation should be maintained in electronic media with suitable backup and for the life of each sample in storage. Specific items to document are listed in Table 2.

Table 2. Documentation requirements for stored urine samples.

Category	**Detail**
Subject pre-analytical variables	Demographics
	Age
	Gender
	Race
	Weight
	Diet
	Time since last meal
	Exercise and time prior to collection
	Lifestyle
	Disease
	Medications
	Fluid-intake
	Stimulation conditions for diagnostic testing
Urine collection conditions	Voided vs. catheter
	Catheter type, duration of placement
	Timed (specify time interval) vs. untimed
	Time since last urination, if known
	Date and time of collection
	Time since last void
	Midstream or other
	Posture
	Cleansing procedure if used
	Brand and catalog number for all containers (the initial cup, any tubes)
Urine handling	Time and temperature
	Clarity of the urine sample
	Dipstick urinalysis results
	Sediment analysis (desirable)
	Specific gravity or osmolality (if included)
	Conditions for timed collection interval
Urine transportation	Time and temperature
	Local processing before transportation
Urine processing	Thawing protocol (if applicable)
	Mixing protocol
	Centrifugation conditions
	Creatinine concentration
	Aliquot volumes, containers and preservatives
Storage	Rate of freezing
	Temperature
Handling during storage	Temperature (continuous monitoring)
	Relocation for defrosting or freezing failure
	Temperature during any handling events
	Thawing and refreezing events

Research Recommendations

The topics listed below need additional investigation for influence on stability of biomolecules stored for long time intervals in a repository. There are country specific issues regarding biospecimen repository storage that need to be considered and addressed. For example, some countries do not permit export of human samples, which may require harmonizing the analyses at different sites, and standardizing and monitoring the collection and storage procedures. It may be desirable to seek international review and endorsement of recommendations for biospecimen repository storage of samples for future research.

A good approach to develop consensus guidelines on urine biorepository practices for topics with sufficient evidence available is to submit a proposal to the Clinical and Laboratory Standards Institute (CLSI). CLSI is a U.S. based global organization that develops standards and guidelines through a consensus process involving stakeholders from the professions, industry and government sectors.

Specific research recommendations follow:

1. Influence of urine residence time in the bladder

2. Influence of fluid intake prior to urine collection

3. Urine preparation conditions to preserve cells, formed elements, biomolecules, to include:
   - Centrifugation conditions most suitable for preservation of different components, e.g., cells, casts, exosomes, endosomes, insoluble matter, general sediment, complete removal of all particulates
   - Preservatives optimal for preservation of various components, e.g., cells and formed elements, exosomes, endosomes, microparticles, proteins, nucleic acids, organic molecules, and inorganic compounds
   - Use of bulking agents to protect biomolecules during freeze-thaw
   - Time from collection to centrifugation, acidification, use of other preservatives, and freezing
   - Temperature and rate of freezing

4. Transportation conditions and cost comparison for solid CO_2 vs liquid nitrogen vapor phase shipping

5. Long term storage conditions
   - Temperature influence on stability for various sample types (pellet, cleared urine, etc.)
   - When is < -150 °C storage needed?
   - Cost effectiveness of < -70 °C vs < -150 °C storage (consider total cost over a 10 or more year period)

- Container requirements (e.g. liquid volume to total volume fraction in a container, lyophilization effects on small volumes, material influence on stability and adsorption of biomolecules)
- Temperature and conditions for storage of centrifugation pellet components

6. Procedures to validate stability of biomolecules during storage, following thawing, and following thaw-refreeze cycles.

7. Biospecimen lifecycle: How long should samples be stored? How many thaw/freeze cycles are acceptable and for what purposes? What are criteria for discarding samples, e.g., if storage conditions have been compromised?

8. Alternative storage conditions
   - Lyophilization
   - Dried on paper
   - Concentrates

9. Consider a meta-analysis of literature on the topics above to establish best practices and clarify specific research questions.

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Group 2: Measurement Analytic Issues Considerations

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The value of using samples obtained from biorepositories for research purposes is often questioned because of the "quality of urine". It is therefore imperative that various aspects of sample collection, processing, handling and storage be considered prior to starting research when such samples are involved. A series of recommendations were made by Group 2 which should facilitate the availability as well as the quality of samples stored for future research. The first major recommendation is that a biorepository should be an integral component of multicenter NIH clinical trials. These trials provide the opportunity to access invaluable specimens which should be useful for future research. The second recommendation is that multiple aliquots of each sample and its fractions should be reposited. In other words, all urine samples to be banked should be fractionated. Figure 1 shows a schematic approach to properly aliquot collected urine samples.

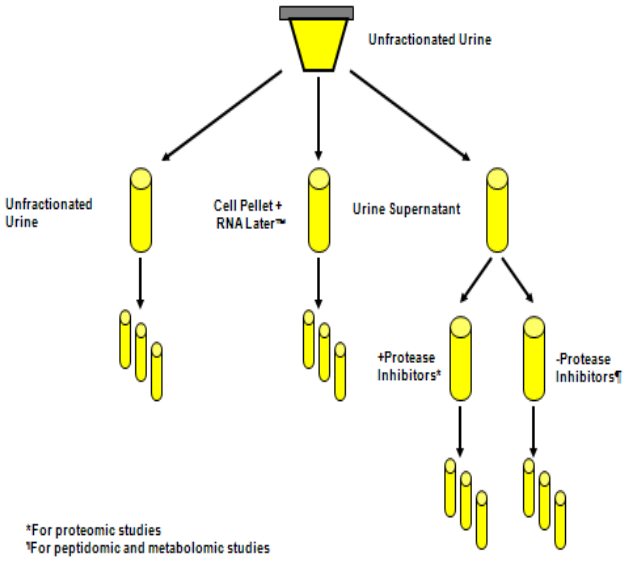

Figure 1. Schematic of urine processing and various aliquots to be stored for each sample. Access to unfractionated urine, cell pellet (for nucleic acid isolation) and addition of protease inhibitors (for proteomic purposes). This approach will assure access to various aliquots of the same sample for use in different research protocols.

The third major recommendation was the development of Sample Operating Procedures (SOP), which is essential for all analytical approaches.

Some of the recommendations regarding sample collection, processing, and handling are general and apply to all three major types of analytes (proteins, nucleic acids and metabolites). For example, no emphasis on diurnal variations or subject preparation (i.e., fasting) should be made. This is because of the impracticality of dictating numerous special conditions when large numbers of banked specimens are to be used for research, especially in large simple studies. Another general issue was the analytical tests being affected by potential interfering compounds or changes in physical parameters (e.g., ionic strength, pH) in highly variable urine samples.

An important and fundamental step to address this challenge is to use matched case and control sets that are treated in the same manner throughout the pre-analytic, analytic, and post-analytic processes. A good way to independently and reliably assess the effect of handling or storage parameters on a particular marker is having access to controls within the same cohort in which the marker of interest is analyzed. Not only are these matched controls useful in accounting for interfering substances, they are also useful to minimize the potentially confounding effects of age, sex, body mass, and urine output in analysis of the values. Therefore, the contribution of each of these variables is initially minimized by using highly matched control sets.

Once a biomarker has been discovered, effects of each of these conditions can be further elucidated in validation studies using larger samples. A major cause of intra-individual variability in biomarker values is related to differences in urine output (hydration), and possible collection of samples in "non-steady state" conditions. The former can theoretically be compensated for by normalization; the latter is more difficult (See Breakout Group 1 section titled "Urine Sample Collection" [near end of last paragraph]). Therefore, in the discovery of new biomarkers, normalization is recommended. The approach and methods need to match the dynamics of the process. In addition, the pathophysiological conditions that affect the analytes need to be investigated.

In addition to the general issues, more specific recommendations were also made based on analyte type. For example, the objective measures to assess the "quality of the urine sample" vary depending on the type of study. It was recommended that the absorbance ratio of 260/280, absolute level of 18S rRNA, TGF-beta, and mRNA copy number be used to assess the quality of the sample for RNA studies. For protein and proteomic analyses, there currently is no clear method to assess integrity of the sample. An approach proposed by the group was to assess the UV absorbance (normalized to urinary creatinine) as a measure of sample integrity and quality. This could perhaps be useful to screen for highly degraded specimens. However, there was disagreement among participants, since other substances in the urine could affect the results of specific evaluations.

A research recommendation is to assess the utility of UV absorbance to assess the quality of urine for protein analysis. In addition, the group also recommended additional research efforts to define measures to assess cysteine and serine protease or phosphatase

activity. The group agreed that there are no known objective quality criteria to guide urine quality assessment for metabolomic and metabolite analyses.

The effects of freeze-thaw cycle and proteases on proteomic analyes of urine are poorly characterized. The sensitivity of proteomic instruments is continuously increasing, and they are able to detect lower levels of analytes and examine them more precisely. Furthermore, it may not be possible to anticipate all effects of freeze-thaw cycle on all types of proteomic studies (e.g., low molecular weight and high molecular weight proteins or peptides, exosomes, etc.). To address the impact of time of storage and freeze-thaw cycles on analytical results, the group recommended that freeze-thaw cycle and protease effects be assessed. A similar approach has been attempted by the NCI's Clinical Proteomics of Technologies for Cancer (CPTC) program. Several studies have outlined approaches to assess urine stability for other types of analytes (such as DNA, mRNA, and microRNA). Such studies should be requested from the investigators seeking to use urine repositories for biomarker studies. Analyte stability is considered to be a critical component in designing sample management strategies. Analyte degradation should be prevented by rapid processing, appropriate storage, and inclusion of inhibitors of enzymes that degrade nucleotides and proteins as guided by the literature. For RNA stability, work by Suthanthiran et al and for exosomes, work by Knepper et al and for metabolomics, laboratory practice guidelines by the National Academy for Clinical Biochemistry may be consulted. Stability data and assessment of degradation for protein markers has changed and is evolving as analytical methodologies become more sensitive. Therefore, it is imperative to consider the methodology used when information and data concerning the stability of proteins are extrapolated from the literature.

Finally, to determine whether very old samples (10-20 years old) currently stored in biobanks may still be use for evaluation of analytes, studies using matched case and control sets seem to constitute the only current practical approach. Such studies will have to be followed by validation of the new findings obtained from the use of older samples in a new set of cases and controls. In addition, in the case of nucleic acids, gross degradation could be determined by UV absorbance. Further research is needed to assess if measuring UV absorbance of the sample is useful in measuring the extent of protein degradation.

In addition, there are several other areas where further research is required to evaluate whether older samples in biobanks are useful for particular research projects. There is a need for a surrogate marker of metabolite stability in stored samples. In other words, is there a metabolite that could be measured, and changes in its concentration be used to assess the extent of degradation of other analytes in the sample? The next approach which needs to be validated for its utility in assessment of sample stability is comparison of current urine albumin concentrations to entry albumin concentrations if similar assays are used.

Group 3: Data Analytic Issues

Josef Coersh, MD (Group Leader)

1. For Data Analysis in Urine Biospecimen Studies:

1. Involve biostatisticians in developing, executing, and writing the data analysis.
2. Incorporate power analyses and multiple comparisons into the interpretation of both positive and negative studies.
3. Quantify sources of variation whenever possible (e.g., analytic, technical, and biologic—day to day and across conditions).
4. Use measures of effect beyond p-values to quantify effects and associations.

2. For Quality Control:

1. Provide detailed documentation of procedures and assays.
2. Standardize conditions whenever possible.
3. Include an assay development plan where appropriate.
4. Incorporate design features to assess different sources of variability
   a) Collection, transport, and analysis: Use blinded split samples collected in the field.
   b) Biologic: Repeat visits more than 48 hours apart.

3. Study Design Issues Important for Defining "Normal" Values for an Analytic Test:

1. Abnormality varies among biomarkers that are qualitatively different when abnormal (bimodal distributions) from the upper or lower end of a continuous distribution.
2. Groups to study
   a) Normal/healthy controls
   b) Subjects with disease
   c) Representative sample of those with another condition who must be distinguished from subjects with the disease
3. Disease biospecimen banks should include controls and cases. Samples from cases and controls should be collected in a similar and standardized manner.

4. "Ideal" normalization criteria and how these affect collection considerations

1. Normalization to urine creatinine is useful
   a) Consider alternative normalization parameters based on platform/analyte and disease type (e.g., RNA for 18S housekeeping gene).
   b) Consider factors that affect the normalization parameter and exclude extremes.
   c) Avoid extreme overhydration or dehydration.

5. **Characterize** variation in a subject by repeat biological samples

    1. Determine whether repeat samples are necessary.
    2. Determine optimal time frame for repeat sampling.
    3. Determine optimal population for repeat sampling.

6. **Monitor assay by:**
    a) Creating reference standards to follow drift.
    b) Creating large specimen banks versus re-sampling controls and consider leveraging with other cohorts.

7. In the absence of true "gold standards" use current clinical diagnostic standards.

8. Understand and identify data necessary for qualification of a biomarker by FDA.

9. Construct informed consent to reflect future analytical use of samples.
    a) Model consents (National Cancer Institute; National Human Genome Research Institute)
    b) Consider creating a centralized IRB review

    Issues currently being debated include:
    a) How and whether to return genetic results. NHLBI and NHGRI workshops have been held to deal with this and related issues
    b) Trans-NIH guidelines and policy relating to specimen collection
    c) The European Commission through the Joint Research Centre (JRC) of Ispra (Varese, Italy) collection of literature on specimen banking

10. To establish consistency in access to samples

    1. Distinguish between custodian and owner (specimens collected under grants generally are owned by the grantee university or by NIH).
    2. Develop plans for succession of custodianship/ownership for collections.
    3. Study the applicability of NCI Best Practices for Biospecimen Resources

Research Recommendations:

1. Develop guidelines for custody of specimens after a clinical trial has ended.
2. Determine if better normalization markers than creatinine exist.
3. Quantify whether these are substantially better than normalization to serum creatinine.
4. Develop statistical methods for handling imperfect gold standards (beyond receiver-operating characteristics).
5. Develop methods to decrease variability in IRB decision-making (e.g., centralized review, reciprocity).

6. Consider if a UBMTA with standardized “options” (e.g., elements, clauses, or definitions) should be widely adopted, and would it speed negotiations?