Journal of Visualized Experiments

Collection, Processing, and Storage Consideration for Urinary Biomarker Research

--Manuscript Draft--

Article Type:	Invited Methods Collection - JoVE Produced Video
Manuscript Number:	JoVE62453R3
Full Title:	Collection, Processing, and Storage Consideration for Urinary Biomarker Research
Corresponding Author:	Catherine Forster UPMC Children's Hospital of Pittsburgh Pittsburgh, PA UNITED STATES
Corresponding Author's Institution:	UPMC Children's Hospital of Pittsburgh
Corresponding Author E-Mail:	forstercs@upmc.edu
Order of Authors:	Nina Kowalewski
	Catherine Forster
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please specify the section of the submitted manuscript.	Medicine
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Pittsburgh, Pennsylvania, United States
Please confirm that you have read and agree to the terms and conditions of the author license agreement that applies below:	I agree to the Author License Agreement
Please provide any comments to the journal here.	2964
Please indicate whether this article will be Standard Access or Open Access.	Standard Access ($1400)
Please confirm that you have read and agree to the terms and conditions of the video release that applies below:	I agree to the Video Release

TITLE:

Collection, Processing, and Storage Consideration for Urinary Biomarker Research

AUTHORS AND AFFILIATIONS:

Nina N. Kowalewski[1] and Catherine S. Forster[1]

[1]Department of Pediatrics, University of Pittsburgh, Pittsburgh, PA

Email Address of co-author:

Nina N. Kowalewski (kowalewskinn@upmc.edu)

Catherine S. Forster (forstercs@upmc.edu)

Corresponding author:

Catherine S. Forster (forstercs@upmc.edu)

KEYWORDS:

urine, biomarkers, urinary tract infection, proteins stability

SUMMARY:

This protocol aims to provide considerations for urine sample collection, processing, and storage for urine tract infection biomarker studies.

ABSTRACT:

There are several urinary proteins that show promise as novel markers of urinary tract infections. The identification of a novel biomarker that has greater predictive accuracy compared to current diagnostic methods has the potential to greatly improve the ability to manage patients with urinary tract infections. However, sample collection, processing, and storage can all potentially impact the results of biomarker research. Understanding the effects of each of these stages on biomarker studies is necessary to inform future, high-quality research in this area, as well as to critically review other studies in this area. Here, the study reviews the literature regarding the effects of each stage of urine sample processing and reports the effects of various conditions on urinary proteins. The protocol will focus on collection techniques, time and temperature of storage, processing techniques, use of reagents, and long-term freezing on biomarker stability. It will focus on proteins but will briefly discuss other materials that may be utilized in biomarker research. In doing so, this protocol will provide a guide to future researchers to assist in the design of urinary biomarkers studies.

INTRODUCTION:

Urinary tract infections (UTI) are one of the most common bacterial infections in both children and adults[1]. While the diagnosis of UTI in some populations can be uncomplicated, it can be more complex in others, such as those with neuropathic bladders[2]. The ability to accurately diagnose UTIs will help improve antibiotic stewardship efforts by decreasing the use of unnecessary antibiotics and potentially aid in the earlier diagnosis of UTI, thus decreasing the risk of morbidity. Given the prevalence of UTIs, there is significant interest in improving the management of this

common infection.

There is an increasing number of novel biomarkers within the literature that show promise in their ability to diagnose UTI[3–7]. However, there are several factors associated with the processing of urine samples that have the potential to alter results. These factors range from collection methods, temperature and duration of short and long-term storage, processing techniques, reagent use, and freeze-thaw cycles[8]. Understanding how changes in each of these can affect biomarker readings is necessary to both critically interpret research within the literature as well as design high-quality studies focused on urine biomarkers.

Here, a narrative review of the literature is provided on the effects of each factor, including collection techniques, short and long-term storage temperature and duration, reagent use, and the effect of freeze-thaw cycles, on proteins that may have utility as urine biomarkers and provide recommendations for optimal processing based on this review of the literature. This protocol will focus on protein biomarkers measured using western blots or ELISAs.

PROTOCOL:

This protocol follows the guidelines of the institution's human research ethics committee. Ensure that the approval is obtained from the institutional review board (IRB) prior to the collection and utilization of biological specimens for research.

1. Collection

1.1. Obtain urine sample in a sterile specimen cup. Decide the type of urine sample, as well as specific inclusion and exclusion criteria, based on the specific study design. For UTI studies, use either the clean-catch method or catheterization to avoid perineal contamination.

1.2. To obtain a clean-catch urine sample, instruct the participants to wipe down the periurethral area with a towelette, void a small amount into the toilet, and then urinate into the specimen cup.

1.3. Instruct women to use their fingers to spread the labia and men to retract their foreskin (if applicable) prior to urination to avoid contamination.

1.4. Record the time of collection.

1.5. Collect the relevant clinical data from each participant, as required by individual study design and research question.

1.6. Consider performing a urinalysis or urine dipstick on each sample prior to processing and storage if this data is not reliably available from the electronic health record.

2. Sample processing and storage

2.1. Process the samples immediately. If this is not possible, store the sample at 4 °C for up to 24 h.

2.2. If samples cannot be stored at 4 °C or need to be stored at 4 °C longer than 24 h, add 0.2 M boric acid or 10 mM NaN_3 to the samples. Check to ensure such reagents are compatible with planned downstream applications.

2.3. Record the duration of time samples spent at 4 °C.

2.4. Centrifuge the samples at 1000–1500 x *g* for 10–20 min. Centrifugation does not need to be at 4 °C.

2.5. Collect the supernatant and aliquot it into separate microcentrifuge tubes.

2.6. Label the tubes with multiple, clear, identifiers (such as the date and sample identification (ID)). Consider using computer-generated barcodes specifically designed for storage of biological samples at -80 °C. If unavailable, ensure that the pen used to label samples is water-resistant.

2.7. Label each freezer box such that each location has a specific code. For this, number each column with a different letter and each row with a number. This will allow for the creation of maps or other guides for easy sample location.

2.8. Freeze the samples immediately at -80 °C. Record the time of freezing.

2.9. Thaw the samples in a 37 °C water bath on the day of measurement to minimize unnecessary storage at either room temperature or 4 °C.

2.10. Record the times and number of additional freeze-thaw cycles for each aliquot.

3. Analysis

3.1. When using commercially available ELISAs, follow the manufacturer's instructions.

3.2. Run the samples in duplicate.

3.3. Identify the expected concentration of the protein of interest to ensure that the protein levels in the samples fall within the range of the kit. If the expected level of protein exceeds the upper standard, dilute the samples.

3.4. After data is obtained from the plate reader (ELISA) or western blot, determine the concentration of each biomarker in the sample manually (not recommended) or using any software.

3.5. Analyze the results. Data analysis depends on the individual study design.

3.6. Consider adjusting biomarker values to account for the urine concentration.

NOTE: Traditionally, biomarker researchers have used urine creatinine as a method of normalization, especially in participants with normal renal function, to account for urine concentration. However, others report that normalization does not make a difference in the results[4]. To overcome this hurdle, some researchers report both normalized and non-normalized results.

3.7. Recommend reporting ranges of times from collection to freezing, as well as the duration of time at 4 °C prior to processing in published manuscripts to allow for interpretation of results in the context of sample processing (**Figure 1**)

4. Effect of various storage conditions on neutrophil gelatinase-associated lipocalin (NGAL).

4.1. Spike fresh urine with 2 ng/mL of recombinant NGAL.

4.2. Aliquot the urine and subject it to different processing and storage conditions.

4.2.1. Centrifuge the urine at 1000–1500 x *g* for 10–20 min. Centrifugation does not need to be at 4 °C. Store at different conditions (20 °C, 4 °C, -20 °C) for 24 h, 48 h, or 72 h.

4.2.2. Store aliquot of the sample at -80 °C for comparison.

4.3. After maintaining the samples in the different conditions as mentioned in step 4.2.1, measure the levels of NGAL in the samples using a commercially available ELISA kit which includes the controls as per the manufacturer's instructions.

REPRESENTATIVE RESULTS:

Centrifugation had a small impact on NGAL levels. Centrifuged samples stored at -80 °C had lower levels of NGAL than non-centrifuged samples (2.17 ng/mL ± 0.32 ng/mL, 2.77 ng/mL ± 0.21 ng/mL). Freeze cycles also had an impact on NGAL levels after the third freeze-thaw cycle. (**Figure 2**). Of the conditions studied (centrifugation, freeze-thaw cycles, and storage temperature), storage temperature had the greatest impact on NGAL levels. Storage at -80 °C immediately after sample collection was the most stable method of sample preservation. Variation was seen in NGAL levels with each of the other storage conditions, with samples stored at room temperature (20 °C) with the largest change in levels. (**Figure 3**)

Taken together, these results indicate how measured protein levels can vary based on the sample processing method, underscoring not only the importance of a consistent and reliable protocol for reproducible results but also the necessity to evaluate the parameters analyzed here for

individual biomarkers.

FIGURE AND TABLE LEGENDS:

Figure 1: Protocol workflow for optimal processing of urine biomarkers. This flowchart illustrates the suggested workflow for processing urine samples for urine biomarkers.

Figure 2: Urine NGAL concentrations in samples subjected to freeze-thaw cycles. Urine NGAL concentrations decrease after the third freeze-thaw cycle. Error bars represent standard deviation. A total of 8 samples were analyzed, run in duplicate for all 4 freeze-thaw cycle conditions.

Figure 3: Urine NGAL concentrations in various storage conditions. Urine samples spiked with recombinant NGAL were stored at 20 °C, 4 °C, -20 °C for 24 h, 48 h, or 72 h. Samples were also immediately frozen at -80 °C for comparison. Concentrations of urine NGAL have a small degree of variation based on freezing temperature and duration. Error bars represent standard deviation. A total of 20 samples were analyzed, run in duplicate for all 10 time and temperature conditions.

DISCUSSION:

The importance of producing consistent and reproducible results is not limited to the success of individual studies but will also enable a better comparison of results within the literature[9]. Variation between studies in key procedural steps can introduce irreversible bias that may affect biomarker signals and their interpretation, which may be responsible for discrepancies among several studies[10]. This demonstrates the need to establish a more standardized approach to processing urine samples for biomarker studies.

The study design of most urinary biomarker studies will dictate the time and method of urine collection. However, when measuring biomarkers in a non-time-sensitive method, we recommend avoidance of a first-morning sample. While first-morning urine samples are considered to supply the most information-rich urinary profile due to their concentrated nature, these samples are not ideal for measuring urine biomarkers as they are susceptible to bacterial and epithelial cell contamination. This is particularly the case for female urine samples: The comparison of female first-void urine to midstream samples demonstrates significantly different proportions of proteins[11]. Thus, a second morning or random midstream urine sample is better suited for studying protein biomarkers[12]. The method of urine collection is also usually dictated by study design. We recommend the use of either clean catch urine or catheterized samples. Residual urine initially collected for routine clinical purposes is often used in UTI studies and is an accepted method of sample collection. However, while the clean catch is a simple procedure, urethral catheterization is invasive. Catheterized urine collection should only be performed by those who have been appropriately trained to do so and done with the approval of the local IRB. We recommend collaborating with clinicians with appropriate training to obtain catheterized urine samples or utilization of residual urine initially collected for routine clinical care.

Samples should be processed right away to preserve the integrity of any markers of interest.

However, this is often not possible; therefore, it becomes necessary to examine the effect of storage conditions, including duration and temperature, on sample stability. In general, storage at room temperature should be minimized as it has been well-founded that prolonged sample storage at room temperature for biomarkers such as NGAL can result in degradation[13]. While some studies have found that 24 h of storage at room temperature (25 °C) has minimal impact on concentration for biomarkers including NGAL and KIM-1[14,15], others have determined that significant degradation does indeed begin to occur within this time frame[8]. The urinary biomarker IL-18 has been found to be particularly sensitive to short-term storage at 25 °C[14,15]. When possible, samples should be stored short-term at 4 °C instead. At 4 °C, NGAL and KIM-1, IL-18, L-FABP, and cystatin C exhibit high stability for 24 h[8] and up to 48 h[15]. However, after 48 h at 4 °C, KIM-1 begins to degrade significantly[16]. Finally, if short-term storage of samples at room temperature exceeds 8 h, or if storage at 4 °C exceeds 16 h, sodium azide or boric acid should be considered as a preservative to inhibit bacterial overgrowth and contamination[12].

Duration of long-term storage is another important consideration. Storage of samples in -80 °C leads to significantly improved stability of urinary proteins compared to storage at -20 °C, which results in significant variability of degradation in proteins such as NGAL[14,13]. At -80 °C, samples can be reliably stored for at least 6 months[16] and up to 2 years[10]. One study found that protein levels had a small but significant decrease after 5 years at -80 °C[8], suggesting that samples should be analyzed prior to 5 years. During long-term storage of samples, freeze-thaw cycles should be minimized to three or fewer[10,11]. Exceeding three freeze-thaw cycles has been found to alter the urine sample[17], which is also confirmed in the data presented here. Of note, for biomarkers without documented stability within the literature, we recommend the researchers to consider performing a pilot stability study prior to planning storage of samples at any temperature other than -80 °C.

There is a lack of consensus around the use of reagents for protein stability. Protease inhibitors are not necessary for urine samples given a relative lack of proteases in urine. This is supported by data demonstrating no difference in the types or amounts of proteins between urine samples stored both with and without protease inhibitors.[18] Further, the addition of a protease inhibitor did not make a difference in urine levels of NGAL, KIM-1, or NAG[10]. While some studies have used protease inhibitors, their use is not standardized throughout the wider urine biomarker literature. Other reagents include those to prevent bacterial overgrowths, such as sodium azide and boric acid. While not traditionally used within the UTI biomarker literature, consideration should be given on the basis of the mechanism of the biomarker of interest. For biomarkers whose levels are associated with the degree of bacteriuria, the use of these reagents may be preferable. The other situation in which these reagents may be considered is when urine cannot be stored at 4 °C for less than 24 h, or must be stored at room temperature for a prolonged period of time prior to processing. Although not commonly used within the UTI biomarker literature, if bacterial overgrowth is a concern and optimal storage conditions are not possible, sodium azide or boric acid can be added to samples.[19]

Centrifugation is an important step to minimize contamination of the urine sample by proteins from other cells (e.g., red blood cells, white blood cells, epithelial cells). Samples should be

centrifuged or filtered rapidly following sample collection. The literature suggests that cells within the urine can begin to lyse within 20–30 min after sample collection, potentially confounding protein measurement[12]. Although rapid centrifugation is frequently not possible, researchers must acknowledge the potential for alternative sources of urinary proteins. Given that pyuria is common in UTIs, this is an important consideration for researchers focused on urine biomarkers in UTI. Samples should not be frozen prior to centrifugation. Indeed, freezing the sample, even with prior centrifugation, may result in the formation of a precipitate of primarily calcium oxalate dehydrate and amorphous calcium crystals upon thawing. In samples kept frozen at -20 °C overnight, Saetun et al. demonstrated that these precipitates might cause an associated decrease in urinary protein levels[20]. Another consideration supporting the recommendation not to freeze non-centrifuged urine is that potential lysis of cells in a non-centrifuged sample may further confound results.

Following centrifugation, the next step is to aliquot the samples. The appropriate number of aliquots of urine to be frozen depends on the individual study design. Aliquoting into several tubes allows for the minimization of freeze-thaw cycles at a later point. The volume of the aliquots depends on the intended downstream applications. Most ELISAs or western blots use a small amount of urine, and therefore a minimum of 0.5 mL is usually sufficient for each aliquot. However, much larger volumes of urine are needed for unbiased methodologies, such as mass spectrometry. We recommend identifying the minimum amount of urine needed for the planned experiments to ensure sufficient volumes.

In this protocol, specific details around the measurement of specific urinary biomarkers were left out as this is highly dependent on individual study design. However, the majority of these biomarkers can be measured using ELISA. The first general step of an ELISA includes coating a 96-well plate with an antibody. Many kits will come with this step already completed, but others require that researchers perform this step. Following coating the plate with antibodies, the next step is to add the standards, samples, and controls. Most kits will provide standards for use in the ELISA. It is important to follow the kit's instructions for standard preparation to ensure the creation of the correct standard curve. Use of negative control is recommended. The next step is to add the detection antibody, followed by the addition of the enzyme conjugate. The final step is to add substrate, often followed by a stop buffer. At this point, the ELISA is ready to be read by a 96-well plate reader. Follow the manufacturer's instructions about how to appropriately configure the plate reader for the ELISA in question. This is especially relevant when interpreting the final results. It is important to note the distribution of the expected standard curve from the manufacturer's instructions appropriately, as this will affect both the fit of the curve and the accuracy of the results. Note the R^2 of the standard curve. This denotes how well the curve fits the standards. A low R^2, below 0.90, suggested inaccurate pipetting, which calls into question the accuracy of the results of the ELISA. The standard curve can then be used to determine the biomarker concentration based on the absorbance generated from the plate reader.

Our protocol is similar to others within the literature that focuses on urinary proteins,[21] with many published studies of urinary biomarkers using similar protocols[6,23–26]. Researchers have varied this protocol using a protease inhibitor[26] or filtrating the urine specimen rather than

centrifugation to adapt the protocol to settings without such equipment[4,28]. A major limitation to creating a single, standard protocol is that various biomarkers have differing properties, which may affect processing considerations. Additionally, downstream applications differ based on study design, which may also affect processing. We have highlighted steps in this protocol to signal researchers of places where consideration may be needed based on specifics of their research design. Finally, this protocol is written with a focus on urine protein biomarkers often investigated in UTI studies. Samples collected for unbiased methodologies, such as mass spectrometry, may require additional considerations[28]. Further, other novel biomarkers, such as RNA markers, will require a distinct protocol for optimal processing and storage. Protocols are elsewhere regarding sample considerations for urobiome studies[29].

ACKNOWLEDGMENTS:

No external funding was obtained for this work. Institutional funds were used to obtain the data in this work.

DISCLOSURES:

None of the authors have any conflicts of interest to disclose.

REFERENCES:

1. Foxman, B. Epidemiology of urinary tract infections: Incidence, morbidity, and economic costs. *American Journal of Medicine*. **113** (1 SUPPL. 1), 5–13 (2002).
2. Forster, C. S., Pohl, H. Diagnosis of urinary tract infection in the neuropathic bladder: Changing the paradigm to include the microbiome. *Topics in Spinal Cord Injury Rehabilitation*. **25** (3) (2019).
3. Gadalla, A. A. H. et al. Identification of clinical and urine biomarkers for uncomplicated urinary tract infection using machine learning algorithms. *Scientific Reports*. **9** (1) (2019).
4. Shaikh, N. et al. Biomarkers that differentiate false positive urinalyses from true urinary tract infection. *Pediatric Nephrology*. **35** (2), 321–329 (2020).
5. Renata, Y., Jassar, H., Katz, R., Hochberg, A., Nir, R. -R., Klein-Kremer, A. Urinary concentration of cytokines in children with acute pyelonephritis. *European journal of Pediatrics*. **172** (6), 769–74 (2013).
6. Forster, C. S., Haffey, W. D., Bennett, M., Greis, K. D., Devarajan, P. Identification of urinary CD44 and Prosaposin as specific biomarkers of urinary tract infections in children with neurogenic bladders. *Biomarker Insights*. **14** (2019).
7. Bitsori, M. et al. Urine IL-8 concentrations in infectious and non-infectious urinary tract conditions. *Pediatric Nephrology (Berlin, Germany)*. **26** (11), 2003–2007 (2011).
8. Schuh, M. P. et al. Long-term Stability of urinary biomarkers of acute kidney injury in children. *American Journal of Kidney Diseases*. **67** (1), 56–61 (2016).
9. Hepburn, S. et al. An analysis of the impact of pre-analytical factors on the urine proteome: Sample processing time, temperature, and proteolysis. *Proteomics - Clinical Applications*. **9** (5–6), 507–521 (2015).
10. Han, W. K., Wagener, G., Zhu, Y., Wang, S., Lee, H. T. Urinary biomarkers in the early detection of acute kidney injury after cardiac surgery. *Clinical Journal of the American Society of Nephrology*. **4** (5), 873–882 (2009).

11. Schaub, S. et al. Urine protein profiling with surface-enhanced laser-desorption/ionization time-of-flight mass spectrometry. *Kidney International*. **65** (1), 323–332 (2004).
12. Thongboonkerd, V. Practical points in urinary proteomics. *Journal of Proteome Research*. **6** (10), 3881–3890 (2007).
13. Grenier, F.C. et al. Evaluation of the ARCHITECT urine NGAL assay: Assay performance, specimen handling requirements and biological variability. *Clinical Biochemistry*. **43** (6), 615–620 (2010).
14. Liu, K.D. et al. Storage time and urine biomarker levels in the ASSESS-AKI study. *PLoS ONE*. **11** (10), 1–9 (2016).
15. Parikh, C. R. et al. Urine stability studies for novel biomarkers of acute kidney injury. *American Journal of Kidney Diseases*. **63** (4), 567–572 (2014).
16. Van De Vrie, M., Deegens, J. K., Van Der Vlag, J., Hilbrands, L. B. Effect of long-term storage of urine samples on measurement of kidney injury molecule 1 (KIM-1) and neutrophil gelatinase-associated lipocalin (NGAL). *American Journal of Kidney Diseases*. **63** (4), 573–576 (2014).
17. Hubel, A., Aksan, A., Skubitz, A. P. N., Wendt, C., Zhong, X. State of the art in preservation of fluid biospecimens. *Biopreservation and Biobanking*. **9** (3), 237–244 (2011).
18. Havanapan, P. O., Thongboonkerd, V. Are protease inhibitors required for gel-based proteomics of kidney and urine? *Journal of Proteome Research*. **8** (6), 3109–3117 (2009).
19. Thongboonkerd, V., Saetun, P. Bacterial overgrowth affects urinary proteome analysis: Recommendation for centrifugation, temperature, duration, and the use of preservatives during sample collection. *Journal of Proteome Research*. **6** (11), 4173–4181 (2007).
20. Saetun, P., Semangoen, T., Thongboonkerd, V. Characterizations of urinary sediments precipitated after freezing and their effects on urinary protein and chemical analyses. *American Journal of Physiology - Renal Physiology*. **296** (6), 1346–1354 (2009).
21. Project, H. K. and U. P. *Standard Protocol for Urine Collection and Storage.*
22. Nickolas, T. L. et al. Diagnostic and prognostic stratification in the emergency department using urinary biomarkers of nephron damage: a multicenter prospective cohort study. *Journal of the American College of Cardiology*. **59** (3), 246–55 (2012).
23. Forster, C. S., Loechtenfeldt, A. M., Shah, S. S., Goldstein, S. Urine neutrophil gelatinase-associated lipocalin in girls with recurrent urinary tract infections. *Pediatric Nephrology*. 1–8 (2020).
24. Forster, C. S. et al. Predictive ability of NGAL in identifying urinary tract infection in children with neurogenic bladders. *Pediatric Nephrology (Berlin, Germany)*. **33** (8), 1365–1374 (2018).
25. Forster, C. et al. Urinary NGAL deficiency in children with recurrent urinary tract infections. *Journal of Pediatric Urology*. **32**, 1077–1080 (2017)
26. Gupta, S., Preece, J., Haynes, A., Becknell, B., Ching, C. Differentiating asymptomatic bacteriuria from urinary tract infection in the pediatric neurogenic bladder population: NGAL as a promising biomarker. *Topics in Spinal Cord Injury Rehabilitation*. **25** (3), 214–221 (2019).
27. Shaikh, N. et al. Host and bacterial markers that differ in children with cystitis and pyelonephritis. *Journal of Pediatrics*. **209**, 146-153.e1 (2019).
28. Harpole, M., Davis, J., Espina, V. Current state of the art for enhancing urine biomarker discovery. *Expert Review of Proteomics*. **13** (6), 609–626 (2016).
29. Jung, C. E. et al. Benchmarking urine storage and collection conditions for evaluating the

female urinary microbiome. *Scientific Reports*. **9** (1), 13409 (2019).

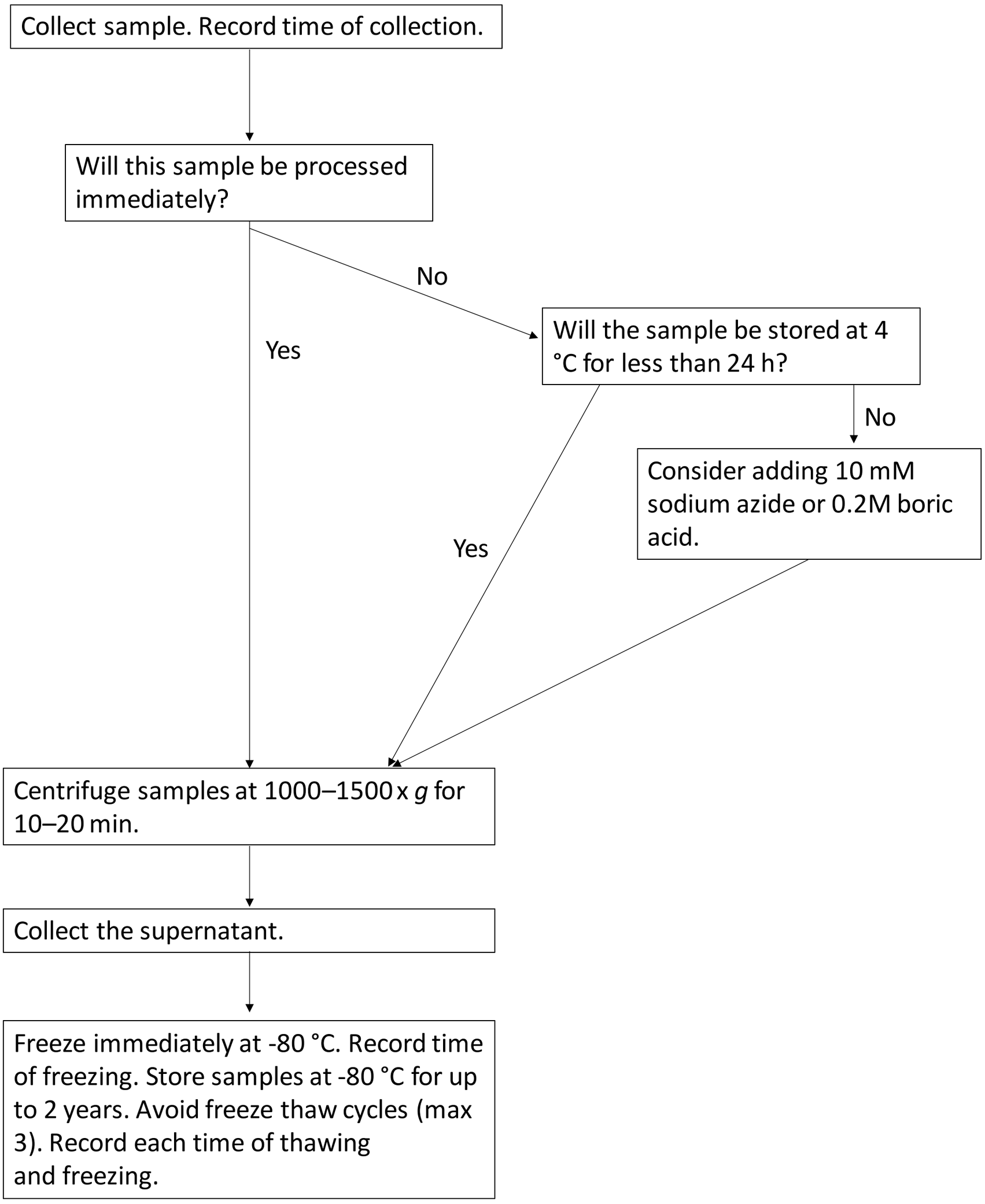


Figure 2

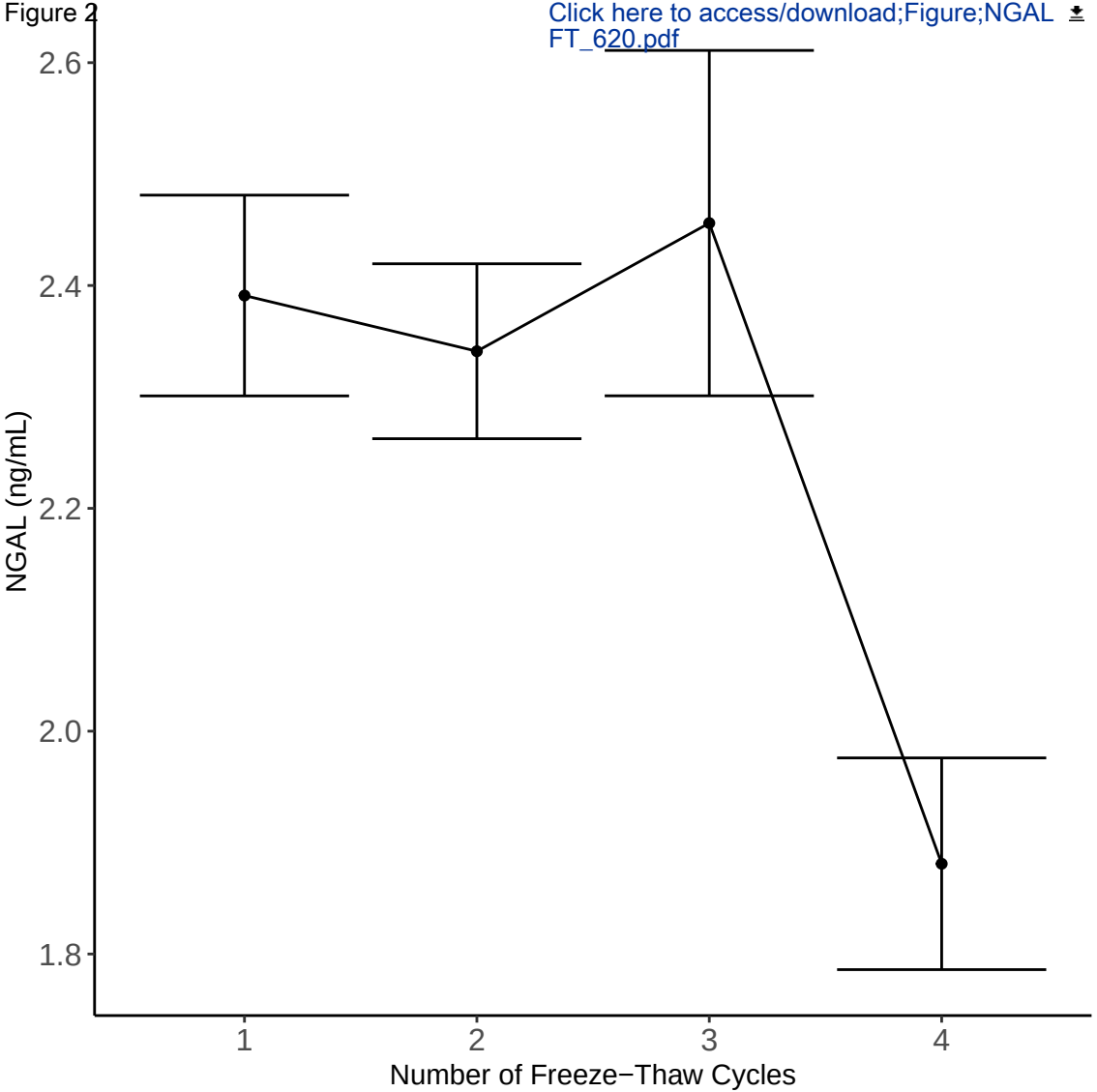
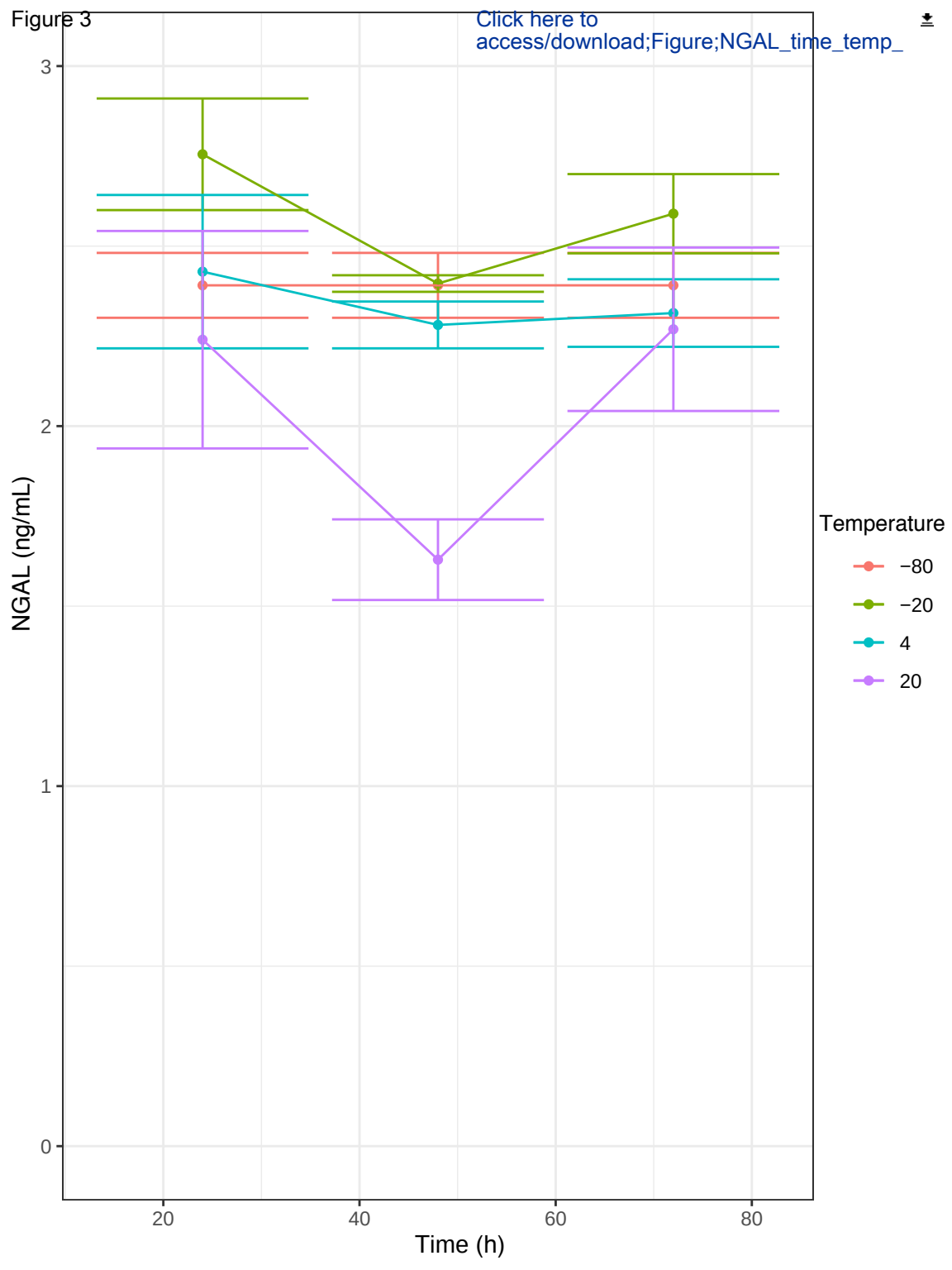


Figure 3


Click here to access/download;Figure;NGAL_time_temp_
3
2
1
0
NGAL (ng/mL)
20
40
60
80
Time (h)
Temperature
−80
−20
4
20

Editor

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

   This has been done.

2. Please focus the manuscript on the protocol instead of a literature review.

   We have emphasized the protocol, as requested by the Editor.

3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials.

   This has been done. We found one use of commercial language within our methods section for the representative results, which was the name of the manufacturer of the ELISA used. We have removed this from the manuscript.

4. Please sort the Materials Table alphabetically by the name of the material.

   This has been done.

5. Please include an ethics statement before the numbered protocol steps, indicating that the protocol follows the guidelines of your institution's human research ethics committee.

   This has been done.

6. Please break up the protocol section into discrete sections that accurately describes the steps: Collection, storage, and processing/analysis, etc.

   This has been done.

7. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

This has been done.

8. The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion

   This change has been made

9. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed?

   We have added as much detail as we believe is necessary to complete the steps in the protocol.

10. Step 1.1.1: What is the patient inclusion/exclusion criteria? Please define second morning urine and catherization or provide references on this. Only mid stream catch is discussed.

    We cannot specify what the appropriate inclusion and exclusion criteria are as this will be highly dependent on the specific question asked by each investigator. We have added the following line to clarify this point:

    > Inclusion and exclusion criteria should be decided based on specific study design.

    We have further edited this point as clean catch and catheterization are the methods used to collect urine, and the other methods (random clean catch and 2nd morning urine) are descriptors of when the sample is collected, not how. Futher, we are hesitant to provide instructions on methods to catheterize a patient as this is an invasive medical procedure. Instead, we have included the following text:

    > Catheterized urine collection should only be performed by those who have been appropriately trained to do so, and done with approval of the local IRB. We recommend collaborating with clinicians with appropriate training to obtain catheterized urine samples, or utilization of residual urine initially collected for routine clinical care.

11. Step 1.1.2: What clinical data is collected? Age/Gender/medical history?

    The clinical data collected is highly reliant on the specific research question and study design. It is beyond the scope of this text to detail what may or may not be appropriate clinical data.

Step 1.1.6: Does centrifugation occur at 4 °C as well?

It does not. We have added the following phrase to address this point:

Centrifugation does not need to be at 4 °C

Step 1.1.7: What are the volumes of the aliquots and the size of the tubes used?

The volume of the aliquots, and corresponding size of the microcentrifuge tube, depends on the down-stream application. We cannot recommend a specific amount, as this depends on individual study design. We have added the following comment to address this point:

The volume of the aliquots depends on the intended down-stream applications. Most ELISAs use a small amount of urine, and therefore a minimum of 0.5 mL is usually sufficient for each aliquot. However, much larger volumes of urine are needed for unbiased methodologies, such as mass spectrometry. We recommend identifying the minimum amount of urine needed for the planned experiments to ensure sufficient volumes.

Step 1.1.8: If removing the compounds here, how is this done?

We have removed this step as it is very uncommon and not done within the urinary biomarker literature that we are familiar with.

Step 1.1.14-1.1.18: Please note that to film these steps, we need more explicit details. The citation of manufacturer's protocols cannot be filmed. ELISA results are presented in the results so this information on how to do so must be explicit in the protocol section.

We request leeway from the Editor regarding this point for several reasons. First, when we created the initial requested representative results, we used a commercial ELISA kit given that this is a very frequent and valid method for measuring urine biomarkers. Second, as each ELISA will be slightly different, we cannot provide a specific protocol that will work for every possible ELISA that a researcher may use. Finally, the focus of this manuscript is intended to be on the processing and storage side of urine samples for biomarker research rather than the measurement side. The current standard within this field of research is to use a validated, commercially-available ELISA to measure urinary proteins. To suggest otherwise would be a disservice to the readers.

11. Please include the details on NGAL usage in the protocol. This is represented in the results.

We used NGAL in the results as it is a common UTI biomarker within the literature, and therefore an appropriate example to be used in the requested result section. Inclusion of NGAL in the protocol is not appropriate as not all UTI researchers will be interested in this biomarker. We have modified the first section of the results to clarify this point:

To demonstrate the effect of various storage conditions on biomarker levels, we measured urine neutrophil gelatinase-associated lipocalin (NGAL) as a representative biomarker of UTI[9] in samples subjected to a variety of storage conditions.

12. Each Figure Legend should include a title and a short description of the data presented in the Figure and relevant symbols. The Discussion of the Figures should be placed in the Representative Results.

    We have provided more of a description of the data in the figure legends.

13. Figure 1 presents a range of concentrations to use (2-20 mM boric acid or 1-10 mM NaN3) but the protocol specifies only one concentration (0.2 M boric acid or 10 mM NaN3). Please homogenize the written protocol details and the figure details.

    This change has been made.

14. Figure 1: For time units, use abbreviated forms for durations of less than one day when the unit is preceded by a numeral. Examples: 24 h, 10-20 min.

    This change has been made.

15. Figure 2: Please include a space after NGAL in the y axis: NGAL (ng/mL) instead of NGAL(ng/mL)

    This change has been made.

16. Figure 2/3: Please use SI abbreviations for milliliters: mL instead of ml.

    This change has been made.

17. Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file. Please sort the Materials Table alphabetically by the name of the material.

    This has been done

18. Regarding filming, please note that we cannot film the collection of the urine due to privacy concerns. Please ensure that the other protocol steps contain enough information to be filmed.

    We completely understand, and agree, that urine collection should not be filmed. We have included sufficient detail in the protocol to help guide other researchers on the

appropriate methods for urine collection and processing when performing biomarker studies. We apologize if this is insufficient for filming, but do not want feel that the inclusion of extra steps for the purpose of filming is warranted.

Reviewer 1:

1. Do the authors recommend obtaining a urinalysis (using dip stick even) prior to adding buffers and storage to be able to have this information on the urine samples?

This is a good suggestion by the review. While the use of the urinalysis data is dependent on study design, we agree that this is likely universally necessary. We have included the following line in the protocol to address this point:

Consider performing a urinalysis or urine dip stick on each sample prior to processing and storage if this data is not reliably available from the electronic health record.

2. Do the authors have a reference for using boric acid or NaN3 for storage?

We have added a reference to this poin in the protocol, and have also added the following line in the discussion to clarify that this is not a common step:

Although not commonly used within the UTI biomarker literature, if bacterial overgrowth is a concern and optimal storage conditions are not possible, , 1–10 mM sodium azide or 2–20 mM boric acid can be added to samples.[9]

3. Can the authors replace "other" on line 124 with "others"

This change has been made.

4. I believe you want to remove "to appropriately" on lines 136-137.

Thank you for pointing this out. We have removed "to appropriately" as suggested.

5. Can the authors clarify "despite prior centrifugation" in line 252? Do they mean "even with prior...."?

We appreciate the reviewer's request for clarity. We have edited the line in question as suggested by the reviewer.

6. Can the authors replace the first "considerations" on line 279 with another word? It seems awkward as written currently.

We have replaced the word "considerations" with "properties".

7.Figures 2 and 3: Can the authors adjust the Y axis label on a scale of 0-3?

This change has been made

Reviewer #2:

1. I think it would be helpful, if the title of the manuscript were more specific and indicated that this review summarizes the methodologies suggested to identify urinary protein biomarkers in urinary tract infections. In fact, line 284 states exactly this, so instead of explaining it at the end of the paper, I would put it in the title or abstract.

We appreciate this point made by the reviewer. We modified the first line of the abstract to explicitly state this, which now reads:

"There are several urinary proteins that show promise as novel markers of urinary tract infections."
We also modified a later sentence in the abstract to read as follows:

"Here, we will review the literature regarding the effects of each stage of urine sample processing and report the effects of various conditions on urinary proteins.

2. Line 99: It would be helpful to suggest a number and volume for the aliquots.

We have added the following line to the protocol to address this point:

*"*The volume of the aliquots depends on the intended down-stream applications. Most ELISAs use a small amount of urine, and therefore a minimum of 0.5 mL is usually sufficient for each aliquot. However, much larger volumes of urine are needed for unbiased methodologies, such as mass spectrometry. We recommend identifying the minimum amount of urine needed for the planned experiments to ensure sufficient volumes. The number of aliquots is also dependent on down-stream uses. Our practice is to reserve a minimum of 2 extra aliquots in the event that validation or other clarification of prior results is necessary.

3. Line 126: Would suggest running all samples in duplicates. Also, would suggest familiarizing oneself with the expected concentration of the protein in the urine to inform need for pre-dilution of the sample, if applicable.

We appreciate this reminder, as we had not included these details in the protocol. We added the following lines to address this point:

*"*We recommend running samples in duplicate. We also recommend becoming familiar with the expected concentration of the protein of interest to ensure that the protein levels within the samples fall within the range of the kit. If the expected level of protein exceeds the upper standard, we recommend dilution of the sample.

4. Line 137: There is a word missing after "appropriately".

This line has been edited.

5. Line 152: Personally, I like to normalize to urine creatinine as it accounts for the concentration of the urine specimen. I would add a sentence to say something to this effect, if the kidney function is normal (then, the urinary creatinine concentration reflects the concentration of the urinary specimen).

We appreciate this point made by the reviewer, and have modified the line in question as follows:

*"*Traditionally, biomarker researchers have used urine creatinine as a method of normalization, especially in participants with normal renal function to account for urine concentration."

1. **The manuscript attached has been formatted to fit the journal standard. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our", etc.).**

   These changes have been made.

2. **The revised version of the submitted manuscript does not have a specific experimental section that can be used for filming. Section 4 has been added to the protocol based on the representative results. Please ensure all the appropriate details like centrifugation condition, the concentration of recombinant NGAL used for spiking, controls, etc., are included in these steps to make it a filmable protocol. Please include the details of the commercial kit used for performing this experiment in the Table of Materials.**

   The details requested above have been added. We have also added the commercial kit to the table of materials.

3. **After including the necessary details to the protocol (section 4), please highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.**

   We have highlighted the portion of the protocol that will be used for filming. As this manuscript was specifically solicited to focus on sample preparation and storage, we have highlighted the relevant section of the protocol. We have specifically not highlighted the measurement section of the protocol as this is outside the scope of the initial purview of this review.

4. **Please include an Acknowledgements section containing any acknowledgments and all funding sources for this work.**

   An acknowledgements section is included.

5. **Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file. Please sort the Materials Table alphabetically by the name of the material.**

   This has been done.

6. **Figure 2/3: Please include the number of samples used for performing the experiment in the Figure Legends section.**

   This change has been made

7. **Please revise the X-axis of Figure 3 to “Time (h)”.**

   This change has been made.