APPLICATION NOTE

# Automation of tissue homogenization for liquid chromatography-mass spectrometry (LC-MS) analysis using the Omni LH 96 automated workstation.


## Summary

Quantifying protein expression via liquid chromatography-mass spectrometry (LC-MS) has become more widely used in all areas of biomedical research and development. Quantifying protein biomarkers can provide essential information on drug efficacy, mechanism of action, target engagement, and safety [1]. In recent years, LC-MS has been applied in a wide array of research areas, including mRNA, lipid nanoparticles (LNPs) [2], gene therapy (GTx) [3], and protein degraders (PROTAC) [4].

LC-MS analysis, when combined with immunoaffinity capture and the use of stable isotope-labeled internal standards, allows for the quantification of protein biomarkers with high sensitivity and unparalleled specificity in complex matrices, including serum, plasma, and tissues. Furthermore, the use of chromatographic separation allows for the quantification of multiple proteins from a single sample. The demand continues to rise to develop and deploy these LC-MS assays quicker than ever, without compromising the consistency and reproducibility of the assay. This has led to a need for automation, particularly in tissue dissection and homogenization, which has been identified as the greatest source of variability during sample preparation for LC-MS [5]. The traditional method for tissue sample preparation (shown in Figure 1) is tedious, time consuming, and labor intensive. It introduces operator variability, making tissue dissection and homogenization an excellent candidate for automation.

This application note focuses on the benchmarking and implementation of the Omni LH 96 workstation to automate the weighing and homogenization of tissue samples for downstream LC-MS analysis. A series of tests will be outlined using various


**Omni LH 96 automated workstation**

tissue types to compare the LH 96 automated workstation vs traditional bead milling. Results showed that the LH 96 was comparable or even more efficient than traditional bead milling in homogenizing various tissue types. LC-MS analysis further proved that the two methods are equally efficient and can be reliably applied in a high-quality assay. Implementation of the Omni LH 96 automated workstation resulted in ~40% increase in throughput. The direct workload for analysts was reduced even greater, as there was no longer a need for tissue sectioning or pulverization, and all weighing, LB addition, and homogenization steps were automated. This frees up hours of analyst time to perform other tasks while the LH 96 workstation is running.

## Materials and methods

### Bead mill and LH 96 homogenization procedures

All tissues were obtained from BioIVT or locally sourced and stored at -80°C until ready for use. The tissues were either pulverized using a crucible (Cell Crusher, Portland OR) or cut into sections, as specified per experiment.

For bead mill homogenization, tissues were transferred to 1.5 mL RINO tubes (Next Advance, Cat # TUBE1R5-S), and a small scoop (about 250 mg) of 0.5 mm diameter stainless steel beads (Next Advance, Cat # SSB05) were added to each sample. Lysis buffer (LB) (5% SDS (Fisher Scientific, Cat # BP2436) in 1x RIPA (Millipore Sigma, Cat # 20-188) with protease inhibitors (Cell Signaling, Cat # 5872) was manually added to yield a concentration of 50 mg of tissue (weight) per mL of LB. Samples were processed in the Omni Bead Ruptor Elite bead mill homogenizer (Revvity, Cat # 19-042E) using a standard method. After centrifugation, the supernatant containing protein was collected and transferred to a clean plate. Samples were used immediately or stored at -80 °C until ready to proceed with downstream analysis.


Figure 1: Traditional bead mill tissue homogenization workflow

For LH 96 samples, prepared tissues were transferred to pre-weighed (using the tare weigh function on the LH 96 system) 14 mL round bottom tubes (Falcon, Cat # 352059) that were compatible with the Omni LH 96 automated workstation (Revvity, Cat # 23-010). Samples were placed onto the LH 96 system and processed using an all-functions profile, including automated sample weighing, net weight calculation, and addition of LB at a concentration of 50 mg of tissue (weight) per mL of LB. Samples were homogenized with the 7 mm Hard Tissue Omni Tip™ plastic homogenizing probes (Revvity, Cat # 30750H) at 18,000 rpm for 45 seconds. Homogenization parameters included a 5 mm cycle height (up and down motion) and a 3 mm side to side motion, to ensure total homogenization. Samples were removed from the LH 96 rack, capped, and centrifuged for 10 min at 4,000 xg at room temperature to reduce foam generated during homogenization. The supernatant containing protein was collected and transferred to a clean plate. Samples were used immediately or stored at -80 °C until ready to proceed with downstream analysis.

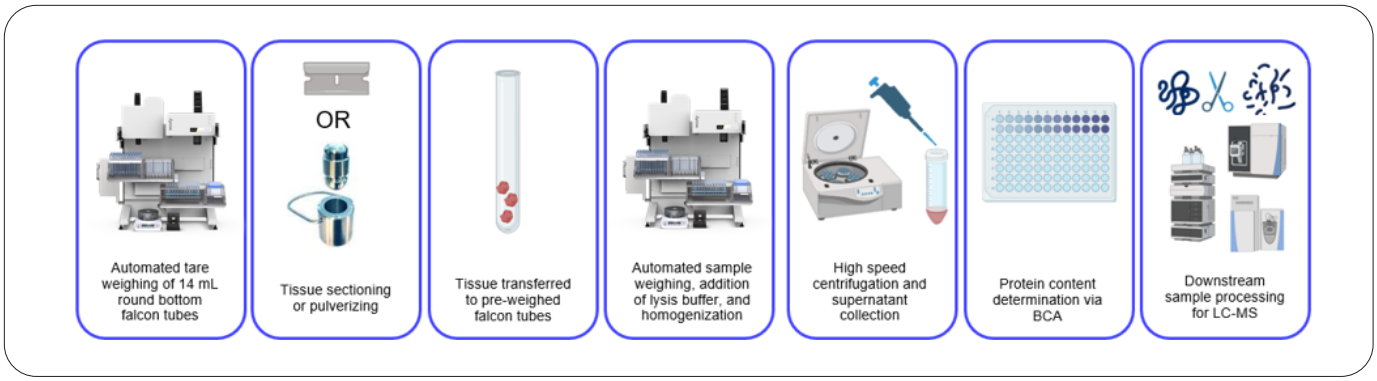


Figure 2: Automated Omni LH 96 tissue homogenization workflow

**Protein content determination via BCA assay**

BCA assay was completed using a Pierce BCA Protein Assay Kit (Thermo Fisher, Cat # 23225) using standard methods. The plate was placed in a Spectra Max i3 plate reader (Molecular Devices, San Jose CA) for absorbance measurement at 562 nm. The total protein was calculated against the calibration curve, fitted using a linear regression in the Spectra Max software.

## Results

**Evaluating weighing precision and accuracy**

Precision and accuracy of sample weighing were evaluated prior to method benchmarking. A significant variability in sample weights was observed for replicate weighting on the Omni LH 96 scale, and a mismatch in weights when compared to an external analytical scale. Figure 3A shows a correlation plot of weights taken with the LH 96 scale and external scale, where the line of best fit had a slope of 0.75, indicating that the LH 96 scale was reading 25% higher than the external scale. The average relative error between weights for these samples was 36%, further proving these measurements were not in agreement. After assessing a variety of causes, including mechanical and user sources, it was determined that interference from static charging was the main source of variability. To reconcile, the scale of the LH 96 was grounded using a grounding mat (Uline, Cat # S-12743) and samples were neutralized with the use of a static removal gun (Millipore Sigma, Cat # Z108812) before processing. With these adjustments, there was an improved correlation of the LH 96 scale with external scale measurements. Figure 3B shows a correlation plot of a set of samples processed after these changes were implemented, resulting in a slope of 1, and a 2% average relative error. This reinforces that it is imperative to ground the scale before processing any samples, as failure to do so will result in increased variability and even incorrect sample weights. After sufficient adjustments were made to address this issue, we were confident to move onto the benchmark testing of the system.

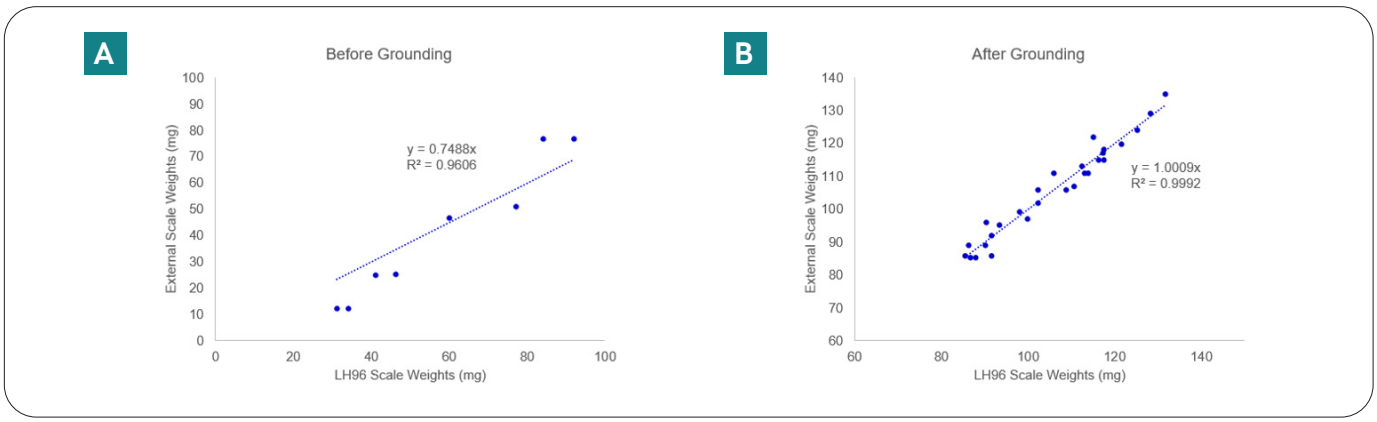


Figure 3: Correlation plots for weights of samples taken on the Omni LH 96 scale versus an external scale. A) Set of samples (N=8) processed before addressing weight inconsistencies. B) Set of samples (N=28) ran after grounding scale to address weight inconsistencies (difference samples from set A)

### Investigating limitations of the system

To begin benchmark testing the system, the first step was to evaluate the efficiency of homogenization for various tissue types. Throughout benchmarking testing, homogenization efficiencies were compared and considered consistent when the percent differences of total measured protein content were less than 20%. Figure 4 shows a comparison of total protein content for homogenization with the Omni LH 96 versus bead milling for three tissue types: rat heart, rat lung, and rat spleen. In the heart and lung samples, the two homogenization methods were consistent, with percent differences of only 4.6% and 4.1%, respectively. In the case of spleen tissue, the LH 96 outperformed bead milling, generating 47% more total protein.

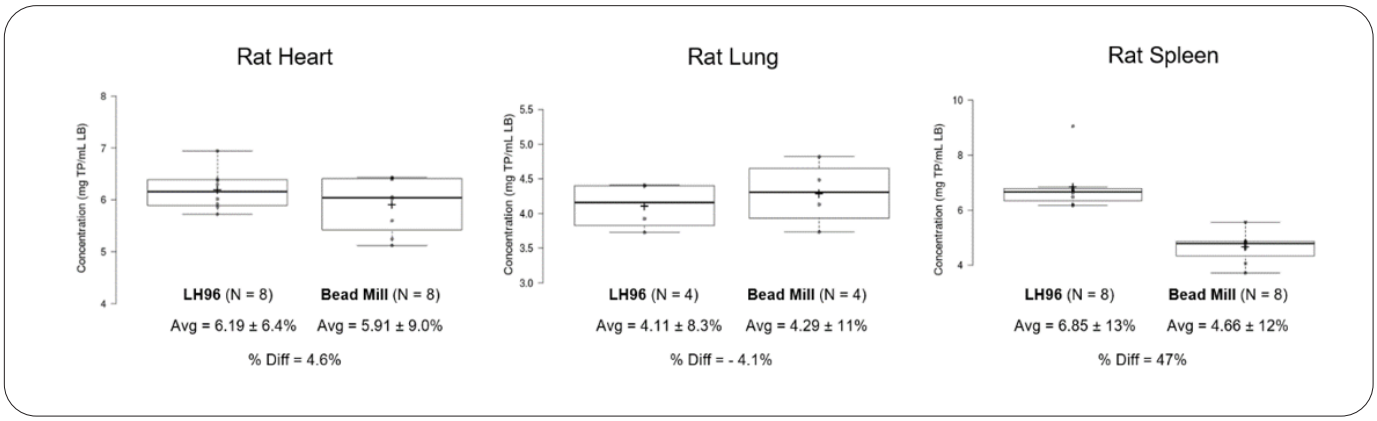


Figure 4: Comparison of tissue homogenization efficiency in different pulverized rat tissues.

In all cases presented in Figure 4, samples were pulverized first before being processed. Pulverization is used to reduce biological variability by increasing the uniformity of the tissue sample, of which a small aliquot can be taken for processing. However, pulverization is the most time consuming and labor-intensive part of the traditional bead milling process, as this is performed one sample at a time in a crucible that requires thorough cleaning between samples. Therefore, homogenizing larger tissue sections, rather than pulverized tissue, was of interest to further reduce analysts' hands-on time. Figure 5 shows that the LH 96 system is more efficient at homogenizing sections of tissue as compared to bead milling. The difference of the means between pulverized and sections of tissue was 80% using bead milling, but when processed on the LH 96 the difference of means was reduced to 12%. This demonstrates that when using the LH 96 system, tissue sectioning rather than pulverization is sufficient. This, along with the increased weight limitations discussed in the next section, allows for the option of whole organ homogenization.

Homogenizing a whole organ accomplishes the same goal of reducing biological variability as pulverization, but with significantly less direct analyst time needed. Whole organ homogenization is possible in cases where the organ weight is within the limitations of the system. Determining these limitations was the next step in benchmarking the system for use.

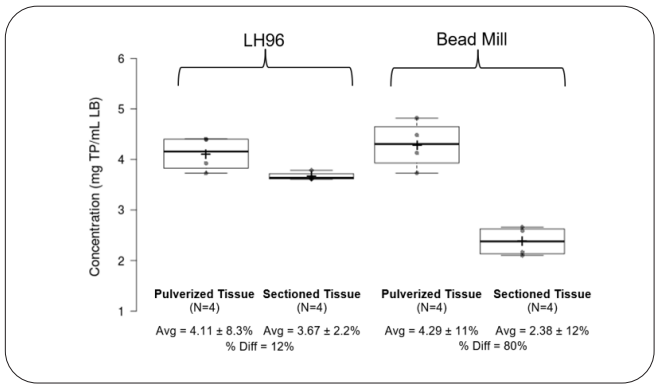


Figure 5: Comparison of tissue homogenization efficiency of pulverized versus sectioned tissues of rat lung tissues, processed on the Omni LH 96 system or the control bead mill method.

To determine the lower limit of sample weights feasible to process, a series of samples with decreasing weights were homogenized and analyzed for total protein content. Tissue sections weighing as low as 10 mg were successfully homogenized and showed consistent homogenization efficiency between LH 96 and bead milling (Figure 6A). For tissues less than 10 mg, insufficient LB volume was dispensed to maintain a 50 mg/mL tissue concentration, which led to incomplete homogenization and inconsistent protein content measurements. Furthermore, the upper weight limit was constrained by the volume of lysis buffer rather than the sample weight itself. The maximum volume for the LH 96 was limited to approximately one half of the tube volume to accommodate for foaming that occurs when using detergent-containing lysis buffers. Figure 6B shows that at 50 mg/mL in a 14 mL tube, up to 400 mg of sectioned tissue with 8 mL of lysis buffer could be homogenized at a consistent efficiency to the control method. This upper limit greatly exceeds the limit of the traditional bead milling method, in which a maximum of 75 mg of tissue can be processed in a 1.5 mL RINO tube. The larger tubes on the Omni LH 96 allow for larger tissue sections to be used, which also increases the number of cases where whole-organ homogenization becomes feasible.

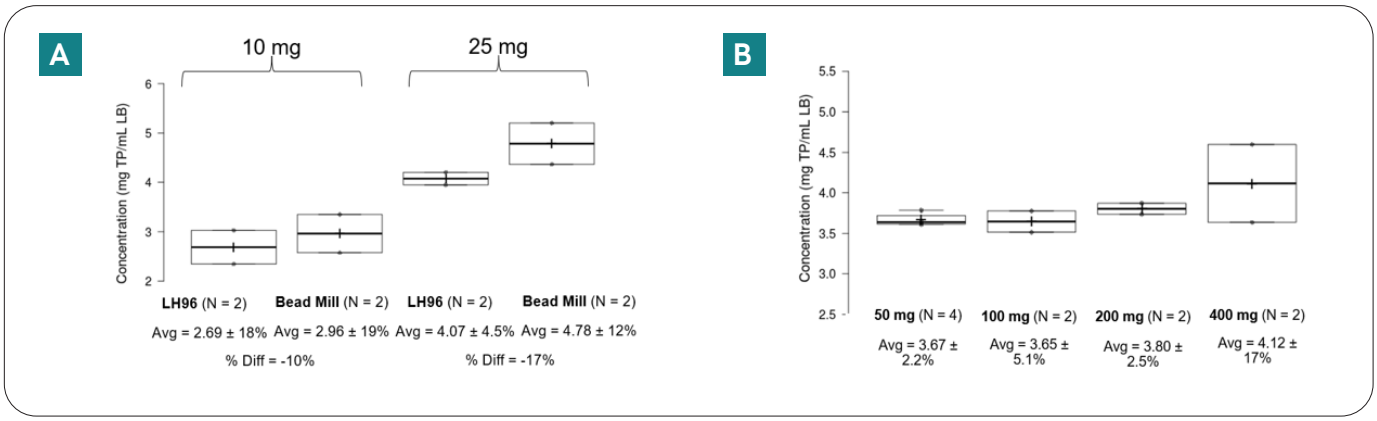


Figure 6A: Tissue homogenization efficiency for a set of pulverized rat liver samples at 25 and 10 mg. 6B: Tissue homogenization efficiency for a set of pulverized rat lung samples at 50, 100, 200, and 400 mg.

Next, the Omni LH 96 system went through a high throughput evaluation. This involved a full continuous run of 96 samples, with the purpose of determining whether technical issues would occur, as well as ensuring consistent homogenization efficiency with a large sample set. In this evaluation, several errors (largely from user error or incorrect setup) were encountered and remedied, until it was possible to complete a full run seamlessly without error. A full set of 96 samples took approximately 2 hours to weigh, add lysis buffer, and homogenize. This is at least a 40% increase in throughput as compared to the traditional bead milling procedure.

From the full set of samples, 28 were selected randomly and analyzed by BCA for total protein. Samples had an average protein concentration of 8.37 ± 11% and 9.03 ± 10% mg TP/mL LB for LH 96 and bead mill samples, respectively. The results were very promising and showed only 8 percent difference of means between the two groups. It is important to note that the LH 96 data set included a few outlying data points. These are likely due to biological variability, as the samples were neither pulverized nor whole organ samples, so there was no control for biological variation. However, when analyzing samples using LC-MS, it is recommended that data is normalized to total protein content which would account for these outliers. To ensure this is true, the last step in benchmarking the LH 96 system was to process and analyze samples with LC-MS to explore any differences during downstream processing.

### Investigating differences from downstream processing

Samples for LC-MS analysis were prepared using an immunoprecipitation workflow to isolate the protein of interest. Briefly, this process used a biotin conjugated antibody to bind the protein of interest, followed by a streptavidin coated magnetic bead that binds to biotin. This streptavidin-biotin-Ab-protein complex was pulled down and the protein was eluted using a strong acid to break the Ab-protein bond. The eluate was a concentrated solution of the protein of interest, which was then digested into peptides for a bottom-up analysis using LC-MS [6]. Using this process, a set of mouse liver samples were processed and analyzed via LC-MS. For this assay, there was a choice between two potential lysis buffers for homogenization, either TPER (Thermo Fisher, Cat # 78510) with 0.2% SDS (Fisher Scientific, Cat # BP2436) or 1% Triton (Thermo Fisher, Cat # 85112) with 0.2% SDS (Fisher Scientific, Cat # BP2436). A set of samples were prepared using each lysis buffer and analyzed for total protein content via BCA (Figure 7). The protein content was slightly lower when prepared with the Omni LH 96, 13% and 20% less than bead milling for TPER and Triton, respectively. While this is a larger difference than seen in previous test cases, it does still meet the criteria set to be considered consistent across both methods. Finally, the samples were analyzed via LC-MS and the signal intensities, normalized by the signal from a heavy-labeled internal standard, were compared. Signal intensities were stable across all samples, regardless of homogenization method. Therefore, even though the protein content of the samples prepared with the Omni LH 96 was slightly lower, the signal response is consistent which reinforces that the LH 96 is recovering proteins of interest. These results strongly indicate that homogenizing samples using the Omni LH 96 automated workstation does not result in any major differences during LC-MS analysis.

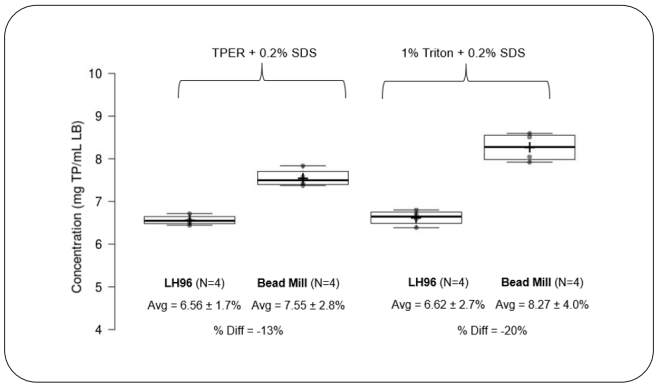


Figure 7: Tissue homogenization efficiency, measured via BCA for total protein content, for Omni LH 96 versus bead milling for two different lysis buffers – TPER with 0.2% SDS and 1% Triton with 0.2% SDS

## Conclusions

The results presented here indicate that homogenizing tissues on the Omni LH 96 automated workstation is equally efficient as traditional bead milling, when using 14 mL round bottom falcon tubes with an SDS containing lysis buffer. The system showed consistent homogenization efficiency for rat heart, lung, and spleen tissues ranging in size from 10 mg to 400 mg, increasing the working range approximately 5 times as compared to the traditional methods. Data presented also showed that homogenization with the Omni LH 96 does not affect downstream analysis of proteins by LC-MS. Future plans include extending capabilities to include more lysis buffer solvents and tissue types, as well as homogenization of tissues for lipid and mRNA analysis.

In conclusion, the LH 96 automated workstation for tissue homogenization is an effective and robust technology that can be implemented to save time and effort when preparing tissue lysates. Use of the system resulted in an estimated 40% reduction of overall processing time needed to prepare samples. More importantly, the amount of direct analyst working time needed is dramatically reduced, freeing up hours of time for the analyst to perform other tasks. Lastly, the user-friendly interface makes it easily transferable between analysts, reducing the time need for training new users. Overall, the Omni LH 96 automated workstation has proved itself to be an essential tool for high-throughput assays.

**Notes: All procedures performed on animals were in accordance with regulations and established guidelines and were reviewed and approved by an Institutional Animal Care and Use Committee or through an ethical review process.**

**All authors declare that they have no conflict of interest, no Pfizer authors have any financial interest in Omni International or any associated companies.**

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