

Centrifugal Microfluidic Automation of Protein Sample Preparation for Nanopore Sensing

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Summary:

We present a microfluidic LabDisk automating the solid-phase-enhanced sample preparation (SP3) workflow for nanopore sensing. Using myelin basic protein (MBP), the system produces nanopore-compatible samples preserving membrane stability and enabling reliable detection with the aerolysin AeL-R220S nanopore variant. A distinct MBP peptide marker supports confident identification. Automated and manual workflows show identical nanopore signals ($I/I_0 \approx 0.75$, $<0.2\%$ deviation) and comparable missed cleavage rates, demonstrating robust, standardized, and user-friendly sample preparation.

Keywords: Proteomics, nanopore sensing, microfluidics, sample preparation automation, SP3

Background, Motivation and Objective

Nanopore technology is a biohybrid single-molecule sensing platform that enables peptide identification through ionic current modulation across a nanoscale membrane pore [1]. Its universal application as a sensor platform is however limited by the need for detergent-free, highly controlled sample preparation, as detergents used in proteomic workflows can destabilize the sensitive membrane. Moreover, in some measurement setups, nanopores operate in highly diluted electrolyte environments, making analyte enrichment a critical prerequisite for reliable detection. SP3 is a magnetic bead-based method that provides an effective strategy for protein sample preparation with efficient detergent removal [2, 3]. Existing SP3 automation approaches based on liquid handling systems [4, 5] improve throughput and reproducibility but rely on complex instrumentation, large reagent volumes and manual intervention during critical steps. We hypothesize that using a microfluidic system with beads confinement in a column format enables precise handling and efficient processing of miniaturized sample volumes, thereby establishing a scalable route toward automation of sample preparation workflows for nanopore biosensing.

Description of the New Method or System

As illustrated in Fig. 1, the manual SP3 workflow and subsequent nanopore measurements are shown. The LabDisk enables full automation of

the SP3 workflow, covering MBP sample processing from denaturation to peptide recovery ($n=1/\text{disk}$). The process begins with manually loading 40 μg MBP sample, bead suspension, digestion mixture and alkylation, reduction mixture into the LabDisk, which is then sealed and inserted into the analyzer. Fluid handling is governed by a combination of thermally induced pneumatic pressure and centrifugal force following a pre-defined protocol. First, denaturation, reduction and alkylation of the protein solution is performed in the incubation chamber, illustrated in Fig. 2. From there, the solution is transferred into the bead column chamber containing 8 μl carboxylated magnetic beads ($>37\ \mu\text{m}$), which are retained by microchannels. Upon release of the binding buffer (acetonitrile) from preloaded stickpacks, it is transferred to the column chamber, where it mixes with the sample and facilitates protein binding to the beads. The bead column is then washed, and the tryptic digestion medium is transferred onto the beads, where digestion is carried out for one hour at 37°C , reducing the total workflow time compared to conventional overnight digestion. Finally the miniaturized peptide eluate ($\sim 14\ \mu\text{l}$) is centrifuged and collected in the outlet chamber from which it can be retrieved.

Results

Nanopore analysis of LabDisk and manually processed samples confirmed full compatibility with nanopore measurements and effective contami-

nant removal by the SP3 workflow. A characteristic peak pattern was observed for both sample types, with peak 4 at $I/I_0 \approx 0.75$ showing $<0.2\%$ deviation across all replicates ($n = 10$ technical replicates from 3 biological samples) (Fig. 3) Spike-in experiments with synthetic peptides identified peak 4 as MBP [H24–R29], establishing it as a unique marker for MBP identification. [3] Data were complemented by LC-MS/MS analysis revealing comparable digestion performance with $40.6 \pm 6.4\%$ (LabDisk) vs. $35.5 \pm 1.3\%$ (manual) missed cleavages, demonstrating equivalent digestion quality. The observed digestion efficiency is consistent with the deliberately shortened 1 h digestion time, chosen to accelerate the workflow. This work demonstrates the proof-of-principle for automated, microfluidic sample preparation enabling reliable peptide identification by nanopore sensing.

Illustrations, Graphs, and Photographs

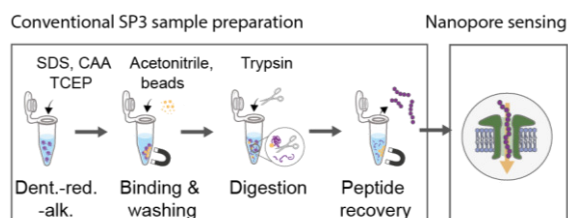


Fig. 1. Schematic of the manual SP3 workflow including denaturation, reduction and alkylation followed by binding, washing, digestion and peptide recovery. The resulting peptides are identified using nanopore sensing. Thereby, a negative voltage is applied across the membrane, causing peptides to translocate electrophoretically through the nanopore. As they pass through, they generate unique current blockades that reflect their physicochemical properties.

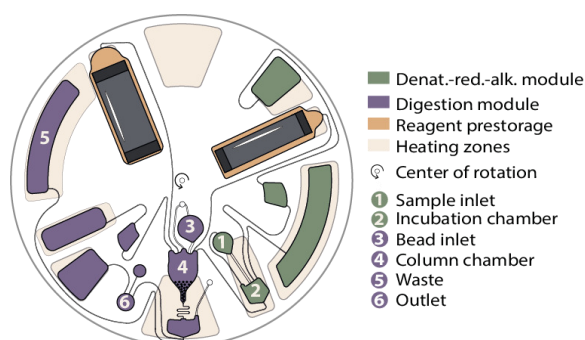


Fig. 2. Illustration of the LabDisk layout comprising modules for denaturation, reduction and alkylation (green), as well as SP3 digestion (purple). Magnetic beads are mechanically retained by filter channels, forming a bead column for solid-phase extraction. Liquid transfer is driven by a combination of thermally induced pneumatic pressure and centrifugal force.

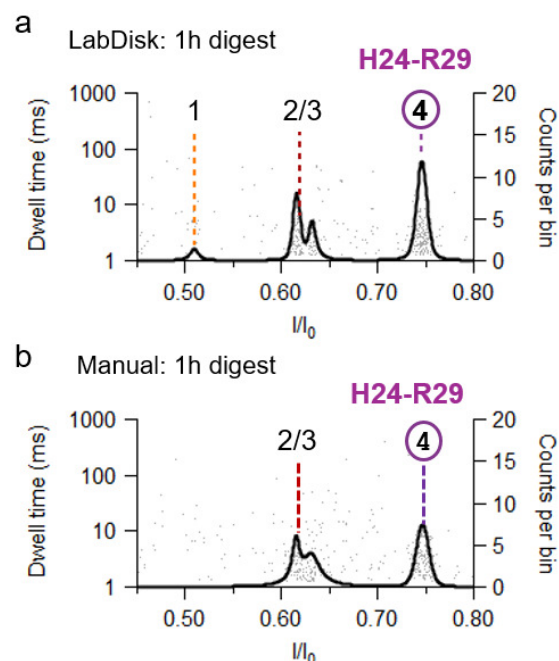


Fig. 3. Nanopore measurements of tryptic MBP digests were performed using the AeL-R220S pore in 4 M KCl, 100 mM Tris (pH 7.5) at -60 mV. Dwell time versus I/I_0 scatter plots with Voigt fits are shown for samples prepared via automated LabDisk and manual workflows. A $5\ \mu\text{L}$ aliquot was analyzed in each case, corresponding to estimated well concentrations of $3.90\ \mu\text{M}$ (LabDisk) and $1.09\ \mu\text{M}$ (manual), reflecting differences in final eluate volumes ($14\ \mu\text{L}$ vs. $50\ \mu\text{L}$).

References

- [1] Afshar Bakshloo M, Kasianowicz JJ, Pastoriza-Gallego M, Mathé J, Daniel R, Piguet F, Oukhaled A., Nanopore-Based Protein Identification, *J Am Chem Soc.*, 144, 2716-2725 (2022); doi:10.1021/jacs.1c11758
- [2] Hughes, C., Moggridge S., Müller T., Sorensen P., Morin G., Krijgsveld J., Single-pot, solid-phase-enhanced sample preparation for proteomics experiments, *Nature Protocols*, 14, 68-85 (2019); doi: 10.1038/s41596-018-0082-x
- [3] Hensel, C, Kleber C, Suppanz I, Gutsch S, Hutz-enlaub T, Behrends JC, von Stetten F, Mittler G, Klatt JN, Schreiber A, Sample preparation for direct nanopore based identification of a biological protein via tryptic peptide recognition, *in prep.*
- [4] Müller T, Kalxdorf M, Longuespée R, Kazdal DN, Stenzinger A, Krijgsveld J., Automated sample preparation with SP3 for low-input clinical proteomics, *Mol Syst Biol.* 16 (2020); doi:10.15252/msb.20199111
- [5] Reder A, Hentschker C, Steil L, Gesell Salazar M, Hammer E, Dhople VM, Sura T, Lissner U, Wolfgramm H, Dittmar D, Harms M, Surmann K, Völker U, Michalik S., MassSpecPreppy-An end-to-end solution for automated protein concentration determination and flexible sample digestion for proteomics applications, *Proteomics*, 24 (2024); doi: 10.1002/pmic.202300294.