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TITLE:

Shotgun Proteomics Sample Processing Automated by an Open-Source Lab Robot

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SUMMARY:

Detailed protocol and three Python scripts are provided for operating an open-source robotic liquid handling system to perform semi-automated protein sample preparation for mass spectrometry experiments, covering detergent removal, protein digestion, and peptide desalting steps.

ABSTRACT:

Mass spectrometry-based shotgun proteomics experiments require multiple sample preparation steps, including enzymatic protein digestion and clean-up, which can take up significant person-hours of bench labor and present a source of batch-to-batch variability. Lab automation with pipetting robots can reduce manual work, maximize throughput, and increase research reproducibility. Still, the steep starting prices of standard automation stations make them unaffordable for many academic laboratories. This article describes a proteomics sample preparation workflow using an affordable, open-source automation system (The Opentrons OT-2), including instructions for setting up semi-automated protein reduction, alkylation, digestion, and clean-up steps accompanying open-source Python scripts to program the OT-2 system through its application programming interface.

INTRODUCTION:

Mass spectrometry-based shotgun proteomics is a powerful tool to measure the abundance of many proteins in biological samples simultaneously. Proteomics experiments with bioinformatics

analysis are routinely employed to identify biomarkers and discover associated biological complexes and pathways underpinning pathological mechanisms. With its high analyte specificity and potential quantitative accuracy, shotgun proteomics also has excellent potential to be adopted by research facilities and diagnostic laboratories for clinical sample analysis without the need to rely on antibodies[12,13].

To prepare protein samples for shotgun proteomics analysis, proteins extracted from biological samples (e.g., cells and tissues) typically first need to be processed using lengthy protocols, including measuring the sample protein concentration, protein reduction, and alkylation, and enzymatic digestion into peptides. Moreover, protein extracted in common lysis buffers containing detergents often requires additional steps of buffer exchange or detergent removal before analysis because detergent can interfere with trypsin digestion and significantly degrade the performance of downstream liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis[1]. Peptides are typically further desalted, dried, and reconstituted in LC-MS/MS compatible solvents following enzymatic digestion. These protein biochemistry procedures can be labor-intensive and time-consuming. Thus, they continue to limit the throughput of proteomics workflows and contribute to the variability of acquired data[2,3]. Human errors and biases have been recognized as crucial factors affecting data variance and reproducibility[4,5]. To minimize human errors in mass spectrometry sample preparation workflows, automated pipetting robotic systems have been utilized to improve the throughput and reproducibility of protein identification and quantification from shotgun proteomics and targeted mass spectrometry analysis, where such advances have been hailed as instrumental for continuing the push for widespread adoption of proteomics technologies in critical research and clinical settings[6–11]. However, most existing protocols utilize robotic liquid handling platforms that require substantial investment and training, limiting their utility in many laboratories in the academic environment or otherwise with a limited budget.

This article describes a protocol that utilizes a low-cost, open-source robotic liquid handling system, the OT-2, to semi-automate a typical shotgun proteomics sample preparation workflow. The OT-2 has a lower cost than many other robotic liquid handling systems, and at the time of writing, costs approximately $5,000 US dollars. When factoring in the prices of different modules and labware, the total cost to set up experiments in this protocol at the time of writing is around $10,000, which renders it more affordable to a considerably broader set of laboratories over more expensive options. The OT-2 is compatible with open-source programming through Python scripts and offers great flexibilities in user-defined DIY protocol design. Using three in-house developed scripts, the protocols below cover executing a typical shotgun proteomics sample preparation workflow on the OT-2 station with an archetypical protein standard (bovine serum albumin; BSA) and a complex protein sample of a normal human heart lysate (**Figure 1**). The procedures for processing (1) a BSA sample and (2) a complex cardiac lysate sample are detailed in Protocol sections 1, 2, 5, 6 and 3, 4, 5, 6, respectively. Sera-Mag carboxylate-modified magnetic beads are utilized in single-pot solid-phase-enhanced sample preparation (SP3) to remove detergents and salts in the protein and peptide samples. Tryptic digests from bovine serum albumin and human heart proteins are further cleaned by SP3 beads and submitted for LC-MS/MS analysis. Mass spectra are then analyzed using the MaxQuant software for peptide and

protein identification. Representative results performed by us show that the protocol achieves excellent technical coefficients of variation (CV) while saving bench time and is non-inferior to hand digest.

PROTOCOL:

The developed Python scripts have been deposited on GitHub at: https://github.com/MaggieLam-Lab/StandardDigestion-Opentrons. A copy of the scripts is given in **Supplementary File 1**. Please refer to the GitHub repository for the latest versions.

1. Experimental preparations

1.1. Check the required hardware before starting the protocol.

NOTE: One needs to check and keep the following ready: OT-2 pipettes, pipette tips, 4-in-1 tube rack set, aluminum block set, magnetic module, temperature module, 96-well 2 mL deep well plates (see **Table of Materials**).

2. Mass spectrometry (MS) sample preparation with a single protein bovine serum albumin (BSA)

2.1. Open the **NoSP3_digestion.py** script in a text editor and specify the experiment-specific variables as needed in the CUSTOMIZE HERE ONLY section.

NOTE: The experiment-specific variables include the number of samples and replicate; the sample concentration; the volume of reagents dithiothreitol - DTT, iodoacetamide - IAA, and trypsin; the DTT and IAA incubation time, and the starting tip for pipette P20/P50 and P300).

2.1.1. Open the Opentrons App and upload the script to the **PROTOCOL** tab in the Opentrons App.

NOTE: The Opentrons App can be downloaded from Reference[14] to a local computer. At the time of writing, the Opentrons P50 electronic pipette is unavailable for purchase in the Opentrons store. It has been replaced with the P20 single-channel electronic pipette compatible with the volumes specified in the protocol. Notes and instructions have been made in the script to replace the P50 pipette with the P20 pipette. Users may need to test and verify the particular pipette's compatibility with this protocol following Opentrons API instruction.

2.2. Open the **ROBOT** tab and perform robot deck calibration **Calibrate Deck** in the **ROBOT** tab following the step-by-step on-screen instructions in the Opentrons App.

NOTE: This step is required only if it has not been previously implemented or the robot has recently been translocated.

2.3. Click on the **MANAGE PIPETTES** button to perform tip length calibration, followed by

pipette offset calibration to calibrate the default tip and pipette combination position.

2.4. NOTE: This step is required if a pipette is used for the first time.

2.5. Place the required labware and pipettes in the corresponding location in the OT-2 deck specified in the Python script (**Figure 2**).

NOTE: Ensure that the temperature module is connected and powered on, and the aluminum block is placed on top of the temperature module.

2.6. Open the **CALIBRATE** tab and perform calibration for the combination of labware and pipettes required in this python script.

NOTE: The Opentrons App will record the calibration parameters, which is not necessary for future applications with the same labware and pipettes.

2.7. Prepare 5 mL of 100 mM ammonium bicarbonate (ABC) (pH ~8.0) solution by dissolving 39.53 mg of ABC in mass spectrometry-grade water to a total volume of 5 mL in a 15 mL conical tube. Place ammonium bicarbonate buffer in the A1 well of the 4-in-1 tube rack with the 15 mL + 50 mL tube holder top.

2.8. Prepare 1 mL of bovine serum albumin (BSA) protein in a 2.0 mL protein low-bind tube (see **Table of Materials**). Place the sample in the A1 well of the 4-in-1 tube rack with 2 mL tube holder top.

2.9. Manually place 2.0 mL protein low-bind tubes in the wells of A1, B1, C1, D1, E1, F1, A2, etc. in the aluminum block on top of the temperature module.

NOTE: The robotic pipette will dispense samples in the vertical order starting from A1 (first sample) until the last sample by default. Horizontal dispensing can be specified. Refer to[15] for details. The total number of tubes that need to be prepared should equal the total number of samples (i.e., the number of biological samples multiplied by the number of technical replicates per sample).

2.10. Prepare 1 mL of 60 mM DTT by dissolving 9.26 mg of DTT solids in mass spectrometry grade water to a total volume of 1 mL. Place the DTT in the A6 well of the 4-in-1 tube rack with the 2 mL tube holder top.

CAUTION: DTT is harmful to human eyes, skin, and the respiratory system. Wear PPE and handle it under a chemical hood. Refer to the manufacturer's safety data sheet for proper procedures.

2.11. Observe while the robot transfers an appropriate volume of ABC buffer to the sample tubes in the aluminum block.

NOTE: The total volume of ABC and protein mix in each tube is 100 µL, and the volume of ABC buffer is calculated in the script (V = 100 µL minus the volume of 100 µg of protein sample).

2.12. Ensure that the robot transfers 100 µg of BSA protein to each tube with ABC buffer.

NOTE: A typical experiment may use up to 100 µg of proteins for protein digestion, i.e., 50 µL of 2.0 µg/µL for this BSA sample.

2.13. Manually verify that the robot program is paused and display the message: Ensure DTT has been loaded into A6 of the 2 mL tube rack located in slot 4 before resuming protocol. Make sure a DTT tube is placed in the A6 well and open its cap. Click on the **Resume** button in the **Opentrons** app to continue. Ensure that the robot transfers 10 µL of DTT solution to each sample well, followed by five mixing rounds.

2.14. Verify that the robot program is paused and display the message: Ensure to close caps on sample tubes. Manually close the caps of the tubes and click on **Resume** to continue. Wait till the robot's temperature module starts to heat the aluminum block until the temperature reaches 55 °C, followed by a 5 min incubation to allow samples to get 55 °C.

NOTE: The robot will hold the temperature at 55 °C for 30 min to allow protein reduction by DTT during incubation.

2.15. During the 30 min of DTT incubation, prepare 1 mL of 187.5 mM Iodoacetamide (IAA) by dissolving 34.68 mg of IAA in the ABC buffer to a total volume of 1 mL. Manually wrap the IAA solution with aluminum foil to avoid exposure to light.

CAUTION: IAA can cause severe eye and respiratory irritation. Handle it under a chemical hood wearing proper PPE.

2.16. Ensure that the robot's temperature module cools down upon completing the 30 min DTT incubation step.

NOTE: After the module's temperature reaches 22 °C, the module maintains the temperature for 5 min to allow the samples to cool down fully.

2.17. Uncap the sample tubes when the robot's program is paused and display the warning message: Ensure to open caps on sample tubes. Click on **Resume** to continue.

2.18. Manually verify that the robot's program is paused with a warning message: Ensure IAA has been loaded into B6 of the 2 mL tube rack located in slot 4 before resuming protocol. Confirm the rack location of the IAA tube and open the tube cap. Click on **Resume** to continue. Ensure that the robot transfers 10 µL of IAA to each sample tube followed by five mixing rounds.

2.19. Cap the sample tubes when the robot's program is paused and display the message: Close

caps on sample tubes and cover sample tubes with foil. Cover the entire aluminum block with a clean piece of foil. Click on **Resume** to continue. Wait till the samples are incubated at 22 °C for 30 min. Ensure that the robot's temperature module deactivates upon completion of IAA incubation.

2.20. Prepare a mixture of trypsin solution during IAA incubation (step 2.19) at the final concentration of 0.2 µg/µL: dissolve 20 µg of mass spectrometry/sequencing-grade trypsin in 100 µL of MS-grade water.

2.21. Place the trypsin solution in the C6 well of the 2 mL tube rack with the tube cap open when the robot's program is paused and displays the warning message: Ensure trypsin has been loaded into C6 of the 2 mL tube rack located in slot 4 prior to resuming protocol. Click on **Resume** to continue.

2.22. Check that the robot's program is paused and display the warning message: Open caps on sample tubes on the temperature module. Uncap the sample tubes and click on **Resume** to continue. Stand by while the robot transfers 10 µL of trypsin to each sample tube followed by five mixing rounds.

2.23. Wrap the sample tube caps with paraffin film, transfer all samples to a temperature-controlled mixer, and incubate at 37 °C for 16–20 h with 600 rpm shaking.

NOTE: The trypsin digestion may also be performed directly on the temperature module at 37 °C.

3. Peptide clean-up using SP3 paramagnetic beads

3.1. The next day after overnight trypsin digestion, briefly spin the samples using a benchtop microcentrifuge (≤2,000 x *g*) (see **Table of Materials**) and place the samples on a magnetic tube rack. Let the samples stand for 2 min.

3.1.1. Transfer the supernatant carefully with a pipette to a new set of protein low-bind microcentrifuge tubes. Keep the samples in a refrigerator and proceed to steps 3.2–3.9.

NOTE: For long-term storage, store the samples at -80 °C.

3.2. Open the **SP3_peptide_cleanup.py** Python script in a text editor and specify as needed in the CUSTOMIZE HERE ONLY section.

NOTE: The experiment-specific variables include the number of samples and replicates, the volume of peptides to be transferred, the volume of reagents (beads, acetonitrile, DMSO), the starting tip for P20/P50 and P300 pipettes, and starting well in the deep-well plate on the magnetic module. Each BSA digest sample contains about 120 µL of digestion volume, which will be aliquoted into two technical replicates with 55 µL in each replicate. To clean up half of the digest only, change the replicate number variable to 1.

3.3. Upload the script to the **PROTOCOL** tab in the **Opentrons** app.

3.4. Place the required labware and pipettes in the corresponding location in the OT-2 deck specified in the python script (**Figure 3**). Make sure the magnetic module is powered on and connected to the robot. Place a new 2 mL 96-well deep well plate (see **Table of Materials**) on the top of the magnetic module.

3.5. Open the **CALIBRATE** tab and perform calibration for the combination of labware and pipettes required in this Python script.

NOTE: Calibration for the same labware and pipette combinations only needs to be performed once, and the Opentrons App will record the calibration parameters.

3.6. Place the digested samples (supernatant collected at step 3.1) to the 2.0 mL tube rack in the vertical order in wells A1, B1….

3.7. Prepare 15 mL of LC-MS/MS-compatible acetonitrile in a 50 mL conical tube and place the tube in the well A3 in the 4-in-1 tube rack with the 15 mL + 50 mL tube holder top.

3.8. Prepare 5 mL of 2% DMSO by adding 100 µL DMSO with 4.9 mL mass spectrometry-grade water in a 15 mL conical tube. Place the tube in the well A1 in the 15mL + 50mL tube rack.

3.9. Label an empty 50 mL conical tube as Waste and place it in the well B3 in the 15mL + 50mL tube rack.

3.10. Prepare the SP3 beads following Reference[16].

3.10.1. Prepare an appropriate amount of mixed beads in a 2.0 mL microcentrifuge tube.

NOTE: Each peptide clean-up reaction requires 10 µL of mixed beads. For example, prepare a minimum of 40 µL of mixed beads for four clean-up reactions.

3.10.2. Let the bead mixture sit on the magnetic stand for 2 min. Remove the supernatant carefully with a pipette and measure the volume of the supernatant with a pipette.

3.10.3. Calculate the remaining volume of beads and add 5–10 times the mass spectrometry grade water (e.g., 100–200 µL water for 20 µL of beads) and vortex (speed 10) for 10 s. Sit the beads on the magnetic stand for 2 min.

3.10.4. Repeat water wash steps for a total of three washes.

3.10.5. Resuspend the final beads in MS-grade water to a final concentration of 50 µg/µL. Place the washed magnetic beads in the well A6 in the 2.0 mL tube rack.

NOTE: The beads are recommended to resuspend at a concentration of 10 µg/µL[16]. In the current optimization effort, the final concentration was modified to 50 µg/µL to minimize the total volume of the bead-peptide-acetonitrile mixture.

3.11. Ensure that the robot transfers 55 µL of the digested samples to the wells in the deep-well plates on the magnetic module.

3.12. Verify that the robot protocol is paused and display the message: Ensure prepared beads have been loaded into A6 of the 2 mL tube rack located in slot 4 before resuming protocol. Vortex the beads, and then briefly spin down for 5 s on a mini benchtop centrifuge and place the beads in the A6 well of the 2 mL tube rack with the cap open. Stand by while the robot mixes the beads 10 times for five rounds by pipetting up and down.

NOTE: The robot transfers 10 µL of beads to each digested sample in the deep-well plates followed by five times of mixing.

3.13. Ensure that the robot transfers 1,292 µL of acetonitrile to each well and immediately mixes 10 times by pipetting up and down to facilitate peptides and beads binding.

NOTE: Each transfer is completed over several rounds because the P300 pipette can only transfer up to 300 µL at a time.

3.14. Ensure that the robot mixes all samples five times by pipetting up and down in the deep-well plate.

3.15. Wait till the magnetic module is engaged and samples are incubated on the module for 2 min.

3.16. Stand by while the pipetting aspiration and dispense speeds are set to slow at 25 µL/s from the default of 150 µL/s.

NOTE: The robot slowly removes the supernatant from each well and discards it in the Waste tube while the magnetic module is engaged.

3.17. Wait till the pipetting aspiration and dispense speeds are returned to the default setting. Observe that the magnetic module disengages.

3.18. Verify that the robot's program is paused and display the message: Make sure the ACN tube cap is off. Manually uncap the acetonitrile tube and place it back to the tube rack. Click on **Resume** to continue. Ensure that the robot transfers 1 mL of acetonitrile to wash each sample and immediately mixes 10 times.

3.19. Ensure that the robot mixes all samples 10 times to wash the samples.

3.20. Stand by while the magnetic module is engaged and incubates the samples for 2 min.

3.21. Wait till the robot changes the pipetting aspiration speed to slow and slowly removes the supernatant and dispenses it in the Waste tube.

3.22. Observe while the robot incubates the samples on the magnetic module for 60 s to allow residual acetonitrile to evaporate. Change pipetting aspiration speeds back to default. Observe that the magnetic module becomes disengaged.

3.23. Verify that the robot's program is paused and display the message: Vortex DMSO again and open caps. Manually vortex the 2% DMSO for 10 s and place it back in the A1 well in the 15 mL–50 mL tube rack. Click on **Resume** to continue.

3.24. Ensure that the robot transfers 80 µL of 2% DMSO to each well and immediately mixes 10 times.

3.25. Ensure that the robot mixes all samples 10 times for additional five rounds.

3.26. Stand by while the magnetic module is engaged and incubates the samples for 2 min.

3.27. Observe while the robot changes the pipette aspiration speed to slow (25 µL/s) and slowly transfers the supernatant to empty wells in the deep-well plate.

3.28. Wait till the robot incubates samples on the magnetic module for 2 min to remove residual beads in the samples.

3.29. Verify that the robot's program is paused and display the message: Place new 2 mL tubes in the 2 mL tube rack and ensure the number of tubes matches the total number of samples.

3.30. Place the first 2 mL microcentrifuge tube in the well directly after the final BSA digest sample. Click on **Resume** to continue.

NOTE: For example, the six BSA digest samples tested in this protocol are in the wells A1, B1, C1, D1, A2, and B2. Therefore, the new set of 2.0 mL tubes is placed in the wells B3, C3, D3, … and so on.

3.31. Observe while the robot transfers 88 µL of samples from the wells in the deep-well plate to the new set of 2.0 mL tubes.

NOTE: The transferred volume (1.1 x 80 µL) in the protocol is optimized to ensure the entire volume of the sample is aspirated into pipette tips.

3.32. Stand by while the robot changes the pipette aspiration speed to default and disengages

the magnetic module.

3.33. Manually dry the cleaned-up peptides in a vacuum evaporator (see **Table of Materials**) and proceed to section 5 or store dried samples at -20 °C.

4. MS sample preparation with protein lysate of the human heart (5 mg/mL) with SP3 paramagnetic beads

4.1. Open the **SP3_digestion.py** Python script and specify values of variables in the CUSTOMIZE HERE ONLY section.

NOTE: The variables include the number of samples and replicate, the sample concentration, the volume of reagents (DTT, IAA, trypsin, beads, 100% and 80% ethanol), DTT and IAA incubation time, starting tip for pipettes P20/P50 and P300, and starting well in the deep-well plate on the magnetic module.

4.2. Follow steps 2.2–2.23 in section 2 for MS sample preparation with a single protein bovine serum albumin for DTT and IAA incubation. Refer to **Figure 1** for the robot deck setup in those steps.

4.3. Prepare a fresh SP3 beads mix (20 µL beads per clean-up reaction) for protein clean-up (as specified in step 3.10) during the DTT and IAA incubation steps (steps 2.15 and 2.19). Place the beads in the D6 well on the 2 mL tube rack.

4.4. Verify that the robot's program is paused with the message: Open tube caps. Manually uncap the sample tubes in the aluminum block on top of the temperature module and click on **Resume** to continue.

4.5. Ensure that the robot transfers all the samples from 2.0 mL tubes to a new deep-well plate on top of the magnetic module.

4.6. Check that the robot's program is paused and display the message: Ensure prepared beads have been loaded into D6 of the 2 mL tube rack located in slot 4 prior to resuming protocol. Open the beads tube cap and click on **Resume** to continue.

4.7. Observe while the robot transfers 20 µL of beads to each well in the deep-well plate with five rounds of mixing of the beads and five rounds of mixing of the sample-beads mixture.

4.8. Verify that the robot's program is paused and displays the message: Ensure 100 percent ethanol has been loaded into A3 of the 15 mL–50 mL tube rack located in slot 5 prior to resuming protocol. Prepare 10–20 mL of 100% ethanol (i.e., 200 proof ethanol, see **Table of Materials**) in a 50 mL conical tube and place it in the A3 well of the rack. Click on **Resume** to continue.

4.9. Stand by while the robot transfers 140 µL of 100% ethanol to each well in the plate

immediately followed by 10 rounds of mixing to facilitate peptides' binding to the beads.

4.10. Ensure that the robot mixes each sample by pipetting up and down 10 times each round for a total of five rounds of mixing.

4.11. Stand by while the magnetic module is engaged and incubates the samples on the module for 2 min.

4.12. Observe while the robot changes the pipetting speed to slow (25 µL/s) and aspirates the supernatant from each well and dispenses it into the Waste tube.

4.13. Wait till the robot changes the pipetting speed back to default and disengages the magnetic module.

4.14. Verify that the robot's program is paused and displays the message: Ensure 80 percent ethanol has been loaded into A4 of the 15 mL–50 mL tube rack located in slot 5 prior to resuming protocol. Manually prepare 20 mL of 80% ethanol by mixing 4 mL of MS-grade water with 16 mL of 100% ethanol (i.e., 200 proof). Place the 80% ethanol in the A4 well in the tube rack. Click on **Resume** to continue. Ensure that the robot transfers 1 mL of 80% ethanol to each well and immediately mixes 10 times.

4.15. Observe while the robot changes the pipetting speed to slow (25 µL/s), aspirates the supernatant from each well, and dispenses into the Waste tube. Wait till the robot changes the pipetting speed back to default and disengages the magnetic module.

4.16. Open the cap of the ABC solution when the robot's program is paused and display the message: Open cap on ABC tube. Click on **Resume** to continue. Stand by while the robot disengages the magnetic module, transfers 250 µL of ABC to each well, and immediately mixes for 10 times.

NOTE: This step is to wash the samples with ABC.

4.17. Ensure that the robot engages the magnetic module and incubates samples on the module for 2 min.

4.18. Observe while the robot changes the pipetting speed to slow (25 µL/s) and transfers the supernatant from each well to the Waste tube. Wait till the robot transfers 100 µL of ABC buffer to each well and immediately mixes for 10 times.

4.19. Verify that the robot's program is paused and displays the message: Ensure new collection tubes have been placed in 2.0 mL aluminum block prior to resuming protocol. Place a new set of low-protein-retention microcentrifuge tubes in the aluminum block immediately after the last sample tube initially in the block. Click on **Resume** to continue. Stand by while the robot transfers each sample in ABC buffer to the new 2.0 mL tubes.

NOTE: Examine the wells and manually transfer any residual sample to the tubes if needed.

4.20. Prepare an appropriate amount of MS-grade trypsin (10 µL per sample) by dissolving 20 µg of MS-grade trypsin in MS-grade water to a final concentration of 0.2 µg/µL when the robot's program is paused and display the message: Ensure trypsin (0.2 µg/µL) has been loaded into C6 of the 2 mL tube rack located in slot 4 prior to resuming protocol. Ensure that the robot transfers 10 µL of trypsin to each sample tube, followed by five rounds of mixing.

4.21. Wrap the sample tube caps with paraffin film, transfer all the samples to a temperature-controlled mixer, and incubate at 37 °C for 16–20 h with 1,000 rpm shaking.

NOTE: Performing the digestion with 1,000 rpm shaking is recommended to minimize beads' precipitation during the overnight incubation.

5. Peptide clean-up using SP3 paramagnetic beads

5.1. Follow stepwise instructions in step 2, Peptide clean-up using SP3 paramagnetic beads.

6. Liquid chromatography and mass spectrometry

6.1. Resuspend the BSA (steps 2–3) and heart lysate (step 4) peptides in 0.1% formic acid by adding 1 mL LC-MS grade 99% formic acid in MS water to a total volume of 1 L.

CAUTION: Formic acid is a strong acid. It is highly corrosive to the eyes, skin, and respiratory system. Handle with care under a chemical hood wearing PPE.

6.2. Quantify the post-digest peptide concentration using a quantitative peptide assay kit[17] and inject 0.5 µg of BSA digest and 1.5 µg of heart digest for LC-MS/MS analysis.

6.3. Set up the liquid chromatography program for LC-MS/MS analysis.

NOTE: In a typical setup, peptide digests may be loaded on a reversed-phase C18 column (3 µm particle; 100 Å pore; 75 µm x 150 mm; see **Table of Materials**) using the parameters provided in **Supplementary File 2**.

6.4. Acquire shotgun proteomics data using a mass spectrometer (see **Table of Materials**) using the parameters provided in **Supplementary File 3**.

6.5. Search the protein database for protein identification.

6.5.1. Download and install the required software, MaxQuant.

NOTE: MaxQuant software (v.1.6.10.43) was used here for the following steps (see **Table of**

Materials).

6.5.2. Download the curated human proteome database from a high-quality protein sequence database (UniProt/SwissProt) (see **Table of Materials**). Click on the **Download** button and choose **FASTA (canonical)**.

NOTE: Optionally, download FASTA (non-canonical) to include protein isoforms of each gene in the database, if desired.

6.5.3. In the MaxQuant software interface, specify the FASTA file to be used as a protein database by navigating to **Global Parameters** panel and click on the **Sequences** tab; then, click on the **Add** button to specify the file path to the FASTA file.

6.5.4. In the MaxQuant software interface, specify the acquired raw mass spectrum files to be analyzed by going to the **Raw Data** panel and clicking on the **Load** button and select the raw file(s).

6.5.5. Set up search parameters as shown in **Table 1**. Enable label-free quantification (LFQ) if needed.

6.5.6. Wait for the search to complete, and locate the number of peptide-spectrum matches (PSMs) that pass the FDR 1% thresholds in the msms.txt file in the /combined/txt folder.

NOTE: In the comparisons performed here for the representative results section, PSMs mapped to multiple proteins were filtered out, and PSMs mapped to one unique protein were retained to count the number of PSMs, peptides, and proteins (**Figure 4** and **Figure 5**).

REPRESENTATIVE RESULTS:

Three Python scripts are provided here that are compatible with the OT-2 robot, and that perform sample preparation for mass spectrometry proteomics with a single protein standard bovine serum albumin (technical replicates n = 5 digestions) and a detergent-containing human heart lysate sample (n = 5 digestions). Each digest product is partitioned into two peptide clean-up reactions. The number of identified peptide-spectrum matches (PSMs), peptides, and proteins in each run of the BSA and heart samples are shown in **Figure 4** and **Figure 5**. A median of 728 PSMs and 65 peptides were identified with the BSA sample, with 5.2% and 3.2% coefficients of variation (CV), respectively. With the complex heart sample, a median of 9,526 PSMs, 7,558 peptides, and 1,336 proteins was identified in 10 runs with 7.6%, 5.9%, and 3.6% coefficient of variation. A total of 1,935 proteins were identified from 10 runs of the heart sample, and among those, 1,677 proteins were identified in two or more runs. To determine the variability in peptide quantification, the CV of the extracted ion chromatogram (XIC) intensities were calculated for 10 peptides that mapped to a unique protein (**Table 2**). The variabilities of human (hand-pipetted) vs. robot experimental results on measuring protein concentration were further compared using three protein standard samples with the BCA assay. The average CV (7.57%) of robot BCA assay was found to be lower than the human manual BCA assay (9.22%) (**Supplementary Table 1**).

The described protocol showed consistent performance over time when the BSA digestion protocol was performed 2 months apart and produced comparable results. The median number of unique PSMs and peptides in **Figure 2** are 728 and 65, respectively. The same experiments performed on the OT-2 system 2 months before generated an average of 647 PSMs and 54 peptides (n = 2) (**Supplementary Table 2**). Longer-term stability may be estimated similarly.

The manual bench processing time (incubation time not included) is calculated between the robot protocol and human processing[18] per sample preparation. With the digestion protocol without detergent removal followed by peptide desalting, the manual processing time is 41 min with the robotic system vs. 61 min by hand. With detergent removal, digestion, and peptide desalting protocol, the manual processing time is 54 min with the robotic system vs. 79 min by hand. Therefore, the semi-automated protocol reduces about 20–25 min of hands-on bench processing time per sample. This time reduction becomes considerable when many samples are processed and may be further improved when multiple OT-2 robots are used in parallel.

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic workflow. Proteins extracted with detergent aid will require processing with an extra step of detergent removal before digestion. Protein samples are digested, and peptides are desalted on the OT-2 robotic system. The peptide digests are injected into a Q-Exactive HF mass spectrometer coupled with a nano-LC. MS spectra are searched against a protein database for protein identification.

Figure 2: Robot deck set up for protein digestion. The specified positions of tip racks, samples, trash, temperature module, and magnetic module are shown. Asterisks denote labware and reagents that are only required for the digestion protocol with detergent removal steps. Boxes with numbers denote unoccupied deck positions.

Figure 3: Robot deck set up for the peptide clean-up script. The specified positions of tip racks, samples, trash, and magnetic module are shown. Boxes with numbers denote unoccupied deck positions.

Figure 4: Number of peptide-spectrum matches (PSMs) and peptides detected in the digestions of BSA protein (n = 5). Each digest was split into two for technical replicate peptide clean-ups (R1 and R2). Coefficient of variations: 5.2% for PSMs; 3.2% for peptides.

Figure 5: Number of PSMs, peptides, and proteins identified from a human heart lysate. Five digestions were performed with SP3 detergent removal. Each digest was split into two for peptide clean-ups (R1 and R2). Coefficients of variation: 7.6% for PSMs; 5.9% for peptides; 3.6% for proteins.

Table 1: The peptide database (MaxQuant) search parameters.

Table 2: Extracted ion chromatogram (XIC) intensity quantification of 10 peptides.

Supplementary Table 1: Comparison of manual and automated BCA assays.

Supplementary Table 2: BSA digestion performed 2 months apart from the samples processed in Figure 4.

Supplementary File 1: A copy of the developed Python scripts.

Supplementary File 2: Method parameters for the liquid chromatography program for LC-MS/MS analysis.

Supplementary File 3: Method parameters for acquiring shotgun proteomics data using a mass spectrometer.

DISCUSSION:

Critical steps within the protocol

For the best performance, Opentrons-verified labware, modules, and consumables should be used compatible with OT-2. Custom labware can be created following Opentrons' instruction at[14]. Make sure to calibrate the OT-2 deck, pipettes, and labware when used for the first time. It is also critical to follow guidelines from SP3 beads' manufacturer to prepare beads for peptide and protein clean-up. Notably, during the bead and peptide binding reaction, the volume of acetonitrile in the binding reaction needs to be ≥95%, and the bead concentration needs to be ≥0.1 µg/µL. Keep the peptide concentration in the range of 10 µg/mL–5 mg/mL. With the optimized parameters in the peptide clean-up script here, the acetonitrile volume ratio is 95%, the bead concentration is 0.37 µg/µL, and the peptide concentration is in the range of 14–37 µg/mL. The peptides mass is estimated to be 40–100 µg in 100 µL of digestion reaction from our experience. For SP3 protein clean-up, 5–10 µg of beads to 1 µg of protein was used and ensured that the minimal beads concentration is 0.5 µg/µL during protein binding. The recommended protein concentration is in the range of 10 µg/mL–5 mg/mL. With the default parameter in the provided script, 1 mg of beads is used for 100 µg protein, and the bead concentration during binding is 3.75 µg/µL, whereas the protein concentration is 0.35 mg/mL.

Modifications and troubleshooting

The default variables in the Python scripts are optimized for standard workflows in our laboratory. Users need to adjust the variables to make the scripts compatible with their applications if required. If the low intensity is observed on MS spectra, protein or peptide loss needs to be checked after each significant protocol section with the protein BCA assay and quantitative peptide assay. When using the protocol on OT-2 for the first time, observe robot handling for each step to ensure the robot performs procedures as expected. At the time of writing, the P50 electronic pipette is no longer available in the Opentrons store. The current script has been modified to indicate where the P20 pipette may be used in its place. Users may refer to the Opentrons API to modify the scripts to use other pipettes, if necessary.

Limitations of the technique

Despite the advantages of using a robotic liquid handling system, caution should be exercised at the performance of fluid transfer between technical replicates. Monitoring the robot during the initial setup and identifying steps is highly recommended. After the robotic liquid transfer, manual recovery of residual volumes may be required to avoid sample loss and reduce variabilities.

Significance with respect to existing methods

This protocol describes a semi-automated mass spectrometry-based sample preparation method using the low-cost and open-source OT-2 liquid handling robot. Very recently, other works have also begun to use OT-2 toward proteomics applications[9]. Compared to existing methods, distinguishing features of this protocol include the use of a relatively low-cost, Python-programmable robot; the incorporation of semi-automated SP3 beads in two steps in the sample preparation protocols, namely, the protein sample detergent removal step and the peptide desalting/clean-up step; as well as the availability of open-source Python scripts to support further development. SP3 Paramagnetic beads bind proteins and peptides efficiently and have been coupled with automated liquid handling systems toward applications in protein clean-up/detergent removal before enzymatic digestion in MS sample preparation[9,11].

Three open-source Python scripts are provided along with this protocol to researchers. The scripts are customizable for individual experimental conditions (e.g., sample number, replicate number, incubation temperature and time, etc.) and allow further development for modified workflows. The protocols afforded an excellent 3%–6% technical CVs in the number of peptides and/or protein identification between MS runs in our laboratory, comparable with previous work on other liquid handling systems (<20%)[7,9].

Future applications

This protocol demonstrates the utility of a low-cost programmable liquid handling system in conjunction with SP3 beads for semi-automated proteomics sample preparation, which can be potentially applicable to mass spectrometry laboratories and core facilities to improve the efficiency of sample processing.

ACKNOWLEDGMENTS:

This work was supported in part by NIH awards F32-HL149191 to YH; R00-HL144829 to EL; R21-HL150456, R00-HL127302, R01-HL141278 to MPL. Figures 1–3 are created with the aid of a web-based science illustration tool, BioRender.com.

DISCLOSURES:

The authors have no conflicts to declare.

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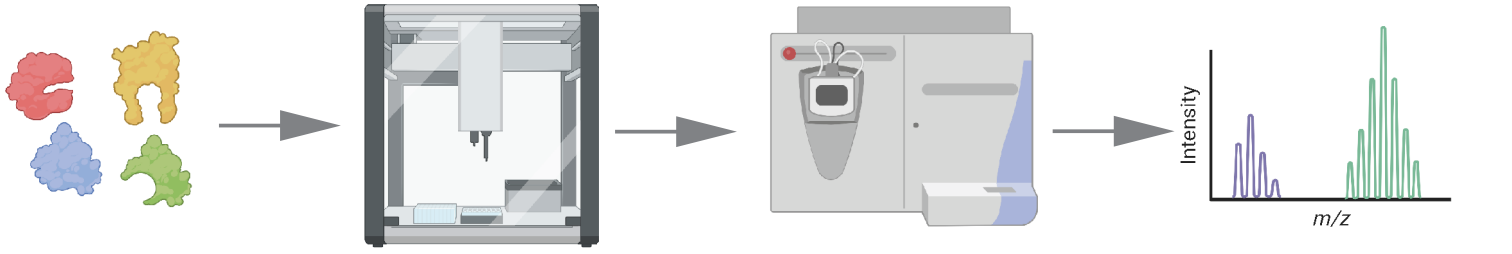
Figure 1



Protein samples

- Detergent removal
- Protein digestion
- Peptide cleanup

Shotgun LC-MS/MS

Protein identification

Figure 2

Figure 3

S1-C1

Figure 4

Click here to access/download;Figure;Figure 4.pdf

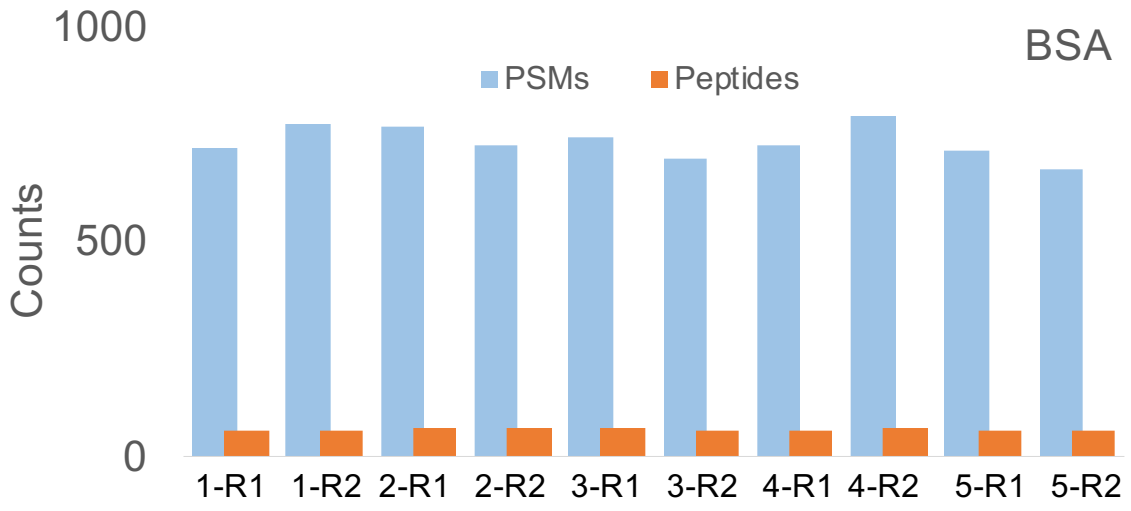
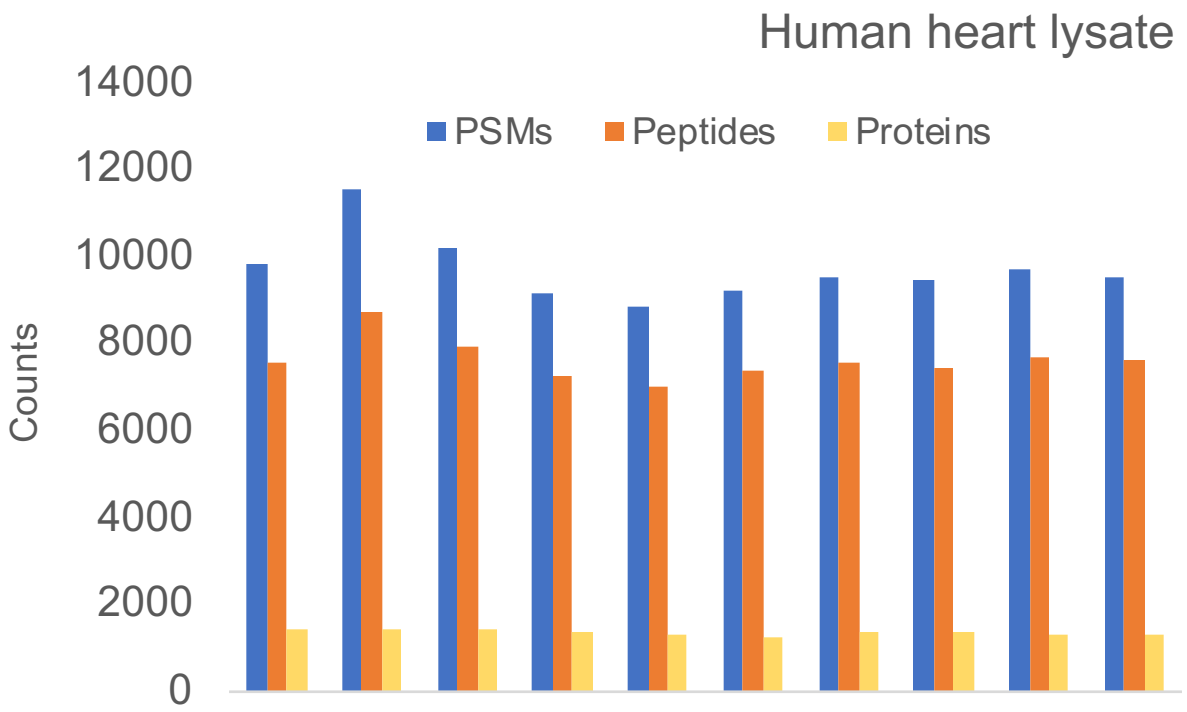


Figure 5

Click here to access/download;Figure;Figure 5.pdf



Digestion enzyme	Trypsin/P
Maximal missed cleavage	2
Fixed modification	Carbamidomethylation of cysteine
Variable modification	N-terminal protein acetylation; oxidation of methionine
Peptide length range	7 – 25 aa
Precursor mass tolerance	± 4.5 ppm
MS/MS ions mass tolerance	± 20 ppm
Label-free quantification	LFQ
False discovery rate (FDR) for peptide-spectrum match (PSM)	0.01

Table 2 Click here to access/download;Table;63092_Table 2.xlsx

Peptide	Protein ID	PEP	Median XIC Intensity
LSTSQIPQSQIR	Q92523	7.72E-08	1.96E+07
SEDFSLPAYMDR	P13073	9.64E-17	8.05E+08
YLQEIYNSNNQK	P02679	2.76E-23	9.69E+08
TDDCHPWVLPVVK	P17174	4.51E-14	4.60E+08
VIVVGNPANTNCLTASK	P40925	7.90E-29	1.17E+09
DYIWNTLNSGR	O75390	1.63E-15	1.38E+08
VSVPTHPEAVGDASLTVVK	P13611	1.86E-09	6.77E+07
QVAEQFLNMR	P22695	3.25E-08	1.09E+08
NTFWDVDGSMVPPEWHR	Q9UI09	2.05E-11	4.00E+07
SASDLTWDNLK	P02787	5.29E-11	1.92E+09

CV
6.70%
7.30%
7.60%
8.60%
8.70%
8.80%
9.10%
9.30%
9.60%
9.80%

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Review Editor
JoVE

September 9, 2021

Dear Dr. Saha,

We would like to submit a revision for our manuscript submission (JoVE63902 “Shotgun proteomics sample processing automated by an open-source lab robot”) for your consideration to be published on JoVE. We thank the Editor for the careful appraisal and review.

In preparing the revision, we believe we have addressed every comment in the manuscript made by the Editor as instructed.

Thank you again for the opportunity to submit our work to JoVE. Please do not hesitate to contact me for matters regarding the manuscript.

Sincerely,

Maggie P Y Lam

Maggie Lam, PhD

Response to Editors' and Reviewers' Comments

Editorial comments:
Changes to be made by the Author(s):

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

Response: Thank you. We have given the manuscript an additional round of proofreading.

2. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Response: We have reviewed the manuscript to avoid the use of personal pronouns where applicable.

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 (including reagents, instruments, software, etc.). Please sort the Materials Table alphabetically by the name of the material.

Response: Thank you. We have checked the manuscript to remove trademarks, registered symbols, and company names preceding reagents.

4. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets or dashes.

Response: We have edited the numbering formats to follow the formatting guidelines.

5. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step.

Response: Thank you. We have checked the protocol to ensure only two to three actions are described in each step.

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

Response: We have revised the protocol and moved appropriate material to the newly reorganized Discussion section (please see Response to Editorial Comment #11).

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. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly.

Response: Thank you. We have modified the protocol to ensure the imperative mood is used, and additional details are marked with “Note”.

8. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

Response: Thank you.

9. In the software, please ensure that all button clicks and user inputs are provided throughout. Also, please ensure that the button clicks are bolded.

Response: We checked the protocol again to ensure user clicks are documented. All button clicks are bolded in the article.

10. Please include one-line space between each protocol step and then highlight in yellow 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.

Response: We have now included a single line spacing in between each step of the protocol. Steps in the protocol that are identified as essential have been highlighted in yellow.

11. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
    a. Critical steps within the protocol
    b. Any modifications and troubleshooting of the technique
    c. Any limitations of the technique
    d. The significance with respect to existing methods

e. Any future applications of the technique

Response: Thank you for the instructions. We have now revised the Discussion section to cover the above topics. Specifically:

Line 622 *"a) Critical steps within the protocol*

For the best performance, Opentrons-verified labware, modules, and consumables should be used that are compatible with OT-2. Custom labware can be created following Opentrons' instruction at https://support.opentrons.com/en/articles/3136504-creating-custom-labware-definitions. Make sure to calibrate OT-2 deck and pipettes, labware when they are used for the first time.

It is also critical to follow guidelines from SP3 beads manufacturer to prepare beads for peptide and protein cleanup. Importantly, during the bead and peptide binding reaction, the volume of acetonitrile in the binding reaction needs to be ≥95%, and the bead concentration needs to be ≥0.1 µg/µL. Keep the peptide concentration in the range of 10 µg/mL to 5 mg/mL. With the optimized parameters in the peptide cleanup script here, the acetonitrile volume ratio is 95%; the bead concentration is 0.37 µg/µL, and the peptides concentration are in the range of 14 – 37 µg/mL. The peptides mass is estimated to be 40–100 µg in 100 µL of digestion reaction from our experience). For SP3 protein cleanup, use 5–10 µg of beads to 1 µg of protein and ensure the minimal beads concentration is 0.5 µg/µL during protein binding. The recommended protein concentration is in the range of 10 µg/mL to 5 mg/mL. With the default parameter in the provided script, 1 mg of beads is used for 100 µg protein and the bead concentration during binding is 3.75 µg/µL whereas the protein concentration is 0.35 mg/mL.

b) Modifications and troubleshooting

The default variables in the Python scripts are optimized for standard workflows in our laboratory. It is important for users to adjust the variables to make the scripts compatible with their applications if needed. If low intensity is observed on MS spectra, check protein or peptide loss after each major protocol section with the protein BCA assay and peptide quantitative assay. For the first time using the protocol on OT-2, observe robot handling for each step to ensure the robot performs procedures as expected. At the time of writing, the P50 electronic pipette is no longer available in the Opentrons store. The current script has been modified to use the P20 pipette in its place. Users may refer to the Opentrons API to modify the scripts to use other pipettes, if necessary.

c) Limitations of the technique

Despite the advantages of using a robotic liquid handling system, caution should be exercised at the performance of liquid transfer between technical replicates. Monitoring the robot during the initial setup and identifying steps is highly recommended, where manual recovery of residual volumes may be required following the robotic liquid transfer to avoid sample loss and reduce variabilities.

d) Significance with respect to existing methods

This protocol describes a semi-automated mass spectrometry sample preparation method using the low-cost and open-source OT-2 liquid handling robot. Very recently,

other works have also begun to use of OT-2 toward proteomics applications[9]. Compared to existing methods, distinguishing features of this protocol include the use of a relatively low-cost, Python-programmable robot; the incorporation of semi-automated SP3 beads in two steps in the sample preparation protocols, namely the protein sample detergent removal step and the peptide desalting/cleanup step; as well as the availability of open-source Python scripts to support further development. SP3 Paramagnetic beads bind proteins and peptides efficiently and have been coupled with automated liquid handling systems towards applications in protein cleanup/detergent removal prior to enzymatic digestion in MS sample preparation.

Three open-source Python scripts are provided along with this protocol to researchers. The scripts are customizable for individual experimental conditions (e.g., sample number, replicate number, incubation temperature and time, etc.) and allow further development for modified workflows. We observed an excellent 3-6% technical CVs in the number of peptide and/or protein identification between MS runs, which are comparable with previous work on other liquid handling systems (<20%)[7,9].

e) Future applications
This protocol demonstrates the utility of a low-cost programmable liquid handling system in conjunction with SP3 beads for semi-automated proteomics sample preparation, which can be potentially applicable to mass spectrometry laboratories and core facilities to improve the efficiency of sample processing."

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
In the manuscript "Shotgun proteomics sample processing automated by an open-source lab robot," Yu Han et al. present a protocol for semi-automated sample preparation utilizing the Opentrons OT-2. Overall, the approach that the authors use - sharing their protocol that can be used with affordable hardware is very laudable, and I am especially fond of the authors sharing their code on GitHub. This general motion towards open science deserves support and will ultimately lead to better and more reproducible science.

Overall, the protocol is easy to follow, and the code is clean, and the authors seem to state realistic expectations of what the advantage of their method is. At the time of reviewing, there were three protocols on OpenTrons related to mass spec, all by far not as extensive as the ones presented here.

Major Concerns:
- None

Response: We thank the Reviewer for reviewing our manuscript and for the encouraging comments. We believe the Reviewer's recommendations have led to an improved revised protocol and manuscript.

Minor Concerns
General:
- It seems that the OT-2 P50 is no longer sold from the vendor. The next best range is either P20 or P300. For the presented scripts, the volumes seem to not exceed 20 uL, so this should not be a problem. However, the authors should add a note for this.

Response: We thank the Reviewer for the reminder. We have now included the note that the P50 is not available for purchase from Opentrons at the moment. As the Reviewer pointed out, the P20 and P300 combination will still cover all needed pipetting volumes. We have modified the scripts to use the P20 pipette by default, and included notes in the protocol (under Step 1.1 and under Discussion) that users may need to modify and verify the exact pipette configuration depending on whether they have the P20 or the P50 pipette.

Line 109: *"Note: At the time of writing, the Opentrons P50 electronic pipette is unavailable for purchase in the Opentrons store and has been replaced with the P20 single-channel electronic pipette which is compatible with the volumes specified in the protocol. Notes and instructions have been made in the script that replaces the P50 pipette with the P20 pipette. Users may need to test and verify the compatibility of the particular pipette being used with this protocol following Opentrons API instruction."*

Line 647: *"At the time of writing, the P50 electronic pipette is no longer available in the Opentrons store. The current script has been modified to use the P20 pipette in its place. Users may refer to the Opentrons API to modify the scripts to use other pipettes, if necessary."*

- Pricing. The base price of the OpenTrons is stated as $5.000. However, the additional modules used in the protocol do cost extra (Magnetic Module $2.400, Temperatur Module $3.000). With pipettes for the robot, a more realistic price estimate would be above $10.000. I think this is still affordable and would recommend the authors highlight these additional costs to generate realistic expectations.

Response: We agree with the Reviewer fully. We have now revised the Introduction section to include the cost of extra modules and consumables as instructed.

Protocol:
- Please add a note to consult the safety data sheet when handling hazardous substances (e.g. DTT)

Response: Thank you for the important reminder. We have now included additional "Caution" notes to remind users to refer to the chemical safety data sheets in relevant steps (1.9 for dithiothreitol, 1.16 for iodoacetamide, and 5.1 for formic acid).

- It would be great if the authors would start with a list of the required Opentrons hardware at the beginning of the protocol.

Response: We have now updated the protocol to include the list of required Opentrons hardware at the beginning. This list is also included in the updated README.md file on the GitHub repo.

Discussion:
-It would be useful to state the total time of manual work in the protocol when performed manually. Otherwise, the 15 to 40-minute speedups are hard to assess. While the speedup might be marginal one could highlight that the process can be parallelized with multiple OT-2s. Furthermore, one would expect the robots to have a smaller variance than humans.

Response: We have now addressed the total time of manual work in the protocol when performed with or without the liquid handling system. We have also highlighted the fact that the time reduction benefits will increase when a larger number of samples are to be processed, or when multiple OT-2s are employed in parallel as suggested. We also now mention the potential for reduced variance of using the liquid handling system, using BCA data in the revised **Supplementary Table 1**. In our hands, the liquid handling system did indeed lead to a small reduction of variance (7.6% CV automated, vs. 9.2% by hand) although we assume that a more accurate estimate of manual CV will require averaging many studies and operators.

Line 584: *"The semi-automated protocol therefore reduces about 20–25 min of hands-on bench processing time per sample. This time reduction becomes considerable when a large number of samples are to be processed, and may be further improved when multiple OT-2 robots are used in parallel."*

Line 568: *"The variabilities of human (hand-pipetted) vs. robot experimental results on measuring protein concentration were further compared using 3 protein standard samples with the BCA assay. The average CV (7.57%) of robot BCA assay was found to be lower than the human manual BCA assay (9.22%) (Supplementary Table 1)."*

-Repeatability. Ideally, it would be great to see how the variance will be when applying the protocol over a longer time period. While maybe no experimental data exists for this, the authors could add a sentence on how they estimate long-term stability (or maybe state that they have successfully used their protocol for X time).

Response: We thank the Reviewer for this helpful suggestion, and have now included representative performance metrics (similar number of peptide and PSM identification) of the protocol performed two months apart in **Supplementary Table 2**. We have revised the text to note that long-term stability may be estimated as suggested by the Reviewer:

Line 573: *"The described protocol showed consistent performance over time when the BSA digestion protocol was performed 2 months apart and produced comparable results. The median number of unique PSMs and peptides in Figure 2 are 728 and 65, respectively. The same experiments performed on the OT-2 system two*

months before generated an average of 647 PSMs and 54 peptides (n=2) (Supplementary Table 2). Longer-term stability may be estimated similarly."

Code:
- The authors should extend their readme on their GitHub page. Specifically, a brief overview of the files can be found. It is to note that the GitHub repository contains 4 *.py scripts, however the protocol describes only 3.

Response: We thank the Reviewer for the suggestion. We have now updated the README.md file in the GitHub repository to include additional information, including an overview of the files, as well as hardware and software requirements. Regarding the number of scripts, three of the .py scripts concern protein digestion as mentioned in the manuscript, whereas the other is used for total protein quantification via bicinchoninic acid assay. This script has now been moved to the "misc_script" folder for clarity. We plan to include additional information and links to the protocol in the near future.

- Exception handling. Some exception handling is performed, but it potentially could be extended. As an example: File SP3_peptide_cleanup.py says for total_samples that it cannot exceed 48. Later there is a check if total_samples + starting_mag_well exceeds 95 and raises an error. As the default starting_mag_well is 0, one could set total_samples to 50 without causing an exception.

Response: We thank the Reviewer for the recommendation. We have now included additional assertions and exception handling in the updated script on GitHub. We plan to continue to update the code to handle additional use cases and scenarios in the future.

- Stars: The GitHub repository currently has 0 stars. I would propose that the authors star their repo to increase visibility.

Response: We thank the Reviewer for the helpful advice. We have starred the GitHub repository, and have plans to coordinate with the potential publication of the article to publicize the repository links on Twitter to further increase visibility.

- Plotting code: If the authors would also share the code they use for plotting the graphs this would be great and in line with the attempt to increase open science.

Response: We thank the Reviewer for the suggestion. We have now included the numerical data used to produce Figure 4 and Figure 5 in the GitHub repository under the example_results subfolder. The data should allow straightforward reproduction of the bar graphs using Python, R, Excel, or other software.

Text:
Sometimes there are symbols that seem to not belong to the text, e.g. L246: "the\ message"

Response: Thank you. This has now been corrected and we have given the manuscript another round of proofreading in preparing the revised submission.

Reviewer #2:

Manuscript Summary:

The manuscript describes a low-cost automated pipetting robotic system anticipated for application in high-throughput proteomics studies. It is well written and straight-forward to follow including a comprehensive discussion and conclusion on the results. Central to the manuscript are detailed protocols how to prepare the samples and how to fill individual wells in laboratory racks that are then loaded into the system. The python scripts as well as the corresponding opentrons python protocol documentation API are very easy to comprehend and adapt for individual purposes.

Response: We thank the Reviewer for the encouraging comments and helpful advice. We are convinced that they have helped us improve our protocol and manuscript.

Minor Concerns:

I have no major concerns and I think this technology could be of great interest to academic proteomics laboratories. In particular JoVe seems to be a nice way to publish and describe this system.

As a small remark to the protocol, while I can not judge the content of each step it seems to be well documented. I would maybe make references in the introduction to the respective protocol numbers BSA sample (Protocol sections 1,2,5,6) and complex human heart lysate (Protocol sections 3,4,5,6) or similar if this is correct to improve readability.

Response: Thank you for the suggestion. We have followed the Reviewer's recommendation and included this in the Introduction section, paragraph 3.

Line 76: *"The procedures for processing (i) a BSA sample and (ii) a complex cardiac lysate sample are detailed in PROTOCOL sections 1, 2, 5, 6 and 3, 4, 5, 6; respectively."*

In addition it would be great if its possible to showcase in the numbers of Table 2 how much less technical variability can be achieved using this system in contrast to manual pipetting work. If such numbers for CVmanual are available.

Response: We thank the Reviewer for the recommendation. Because manual CV is likely influenced by a large number of factors including operator experience, a more complete treatment will likely require a community survey or large-scale meta-analysis of the existing literature. Nevertheless, we have now included the new **Supplementary Table 1**, which shows circumstantial evidence of the potential for reduced variance of using the liquid handling system for BCA assays (7.6% CV automated, vs. 9.2% by hand), at least in our hands. Thank you again for the suggestion.

Supplementary Table 1

Protein standards	2000 μg/mL
Median measured protein concentration with robot BCA assay (n=6)	1862.16 μg/mL
CV of robot BCA	3.21%
Average CV of robot BCA	7.57%
Median measured protein concentration with human manual BCA assay (n=6)	1961.34 μg/mL
CV of manual BCA	6.17%
Average CV of manual BCA	9.22%

1000 µg/mL	500 µg/mL
1064.73 µg/mL	512.31 µg/mL
7.86%	11.65%
1099.89 µg/mL	486.07 µg/mL
17.41%	4.08%

Supplementary Table 2

	Unique PSMs	Unique Peptides
BSA Robot R1	623	53
BSA Robot R2	672	55
Average	647	54

Supplementary File 1

A copy of the Python scripts described in the protocol is included below. Please refer to the GitHub repository for the latest versions.

NoSP3_digestion.py

```
from opentrons import protocol_api

metadata = {
   'protocolName': 'Digestion Protocol 2mL Tubes',
   'author': 'Cody',
   'description': 'Digestion protocol for use with 2mL tubes',
   'apiLevel': '2.8'
}


def run(protocol: protocol_api.ProtocolContext):
   # ---------------------------- CUSTOMIZE HERE ONLY ---------------------------- |
   number_of_samples: int = 1  # specify the number of protein samples
   sample_concentrations = [2.0]   # specify the concentration of protein samples (unit is ug/uL);length of sample_concentrations list must match the number of the samples above;separate concentrations with period sign if sample n>1 (e.g. sample_concentrations=[2.0, 2.5] if sample n=2).
   replicates: int = 9        # specify the number of replicates
   volume_of_DTT: float = 10.0  # manually prepare 60mM DTT in MS-grade water
   volume_of_IAA: float = 10.0  # manually prepare 375mM IAA in MS-grade water
   volume_of_trypsin: float = 10.0  # manually prepare to a concentration of 0.2ug/uL
   incubation_time_DTT = 30  # in minutes
   incubation_time_IAA = 30  # in minutes
   starting_tip_p50 = 'A1'  # change if full tip rack will not be used
   starting_tip_p300 = 'A1'  # change if full tip rack will not be used

   # | ---------------------------- ^^^^^^^^^^^^^^^^^^^^ ---------------------------- |
   # ---------------------------- DO NOT EDIT BELOW THIS LINE ---------------------------- #
   # Check for valid inputs
   if len(sample_concentrations) != number_of_samples:
      raise ValueError('Length of sample_concentrations must match the integer specified for number_of_samples.')
   if number_of_samples * replicates > 24:
      raise ValueError('Total digests (including replicates) cannot exceed the number of slots available on the aluminum block (24).')
```

```
    # | ---------  tip racks --------- |
    tiprack_300 = protocol.load_labware('opentrons_96_tiprack_300ul', 2)
    tiprack_50 = protocol.load_labware('opentrons_96_tiprack_300ul', 1)

    # | ---------  pipettes --------- |
    p300 = protocol.load_instrument('p300_single', 'right', tip_racks=[tiprack_300])
    p50 = protocol.load_instrument('p50_single', 'left', tip_racks=[tiprack_50]) ##change p50 to p20 if p20 will be used here and throughout the script following OT-2 API; this script has not been tested with p20 therefore testing is required.”
    p50.starting_tip = tiprack_50.well(starting_tip_p50)
    p300.starting_tip = tiprack_300.well(starting_tip_p300)

    # | ---------  tube racks/plates/containers --------- |
    temp_mod = protocol.load_module('Temperature Module', 10)
    temp_plate = temp_mod.load_labware('opentrons_24_aluminumblock_nest_2ml_snapcap')
    tuberack_2mL = protocol.load_labware('opentrons_24_tuberack_nest_2ml_snapcap', 4)
    tuberack_15ml_50ml = protocol.load_labware('opentrons_10_tuberack_falcon_4x50ml_6x15ml_conical', 5)

    # | --------- reagents --------- |
    DTT = tuberack_2mL['A6']
    IAA = tuberack_2mL['B6']
    trypsin = tuberack_2mL['C6']
    ABC = tuberack_15ml_50ml['A3']
    samples = tuberack_2mL.wells()[:number_of_samples]

    # ---------------------------- COMMANDS ---------------------------- #

    # | --------- transfer samples to plate --------- |
    protocol.pause('Ensure to change starting tip position for p50 and p300.')

    for i in range(number_of_samples):
        # transfer ABC; change 50 to 20 if p20 will be used.
        if (100 - (100 / sample_concentrations[i])) > 50:
            p300.transfer(
                100 - (100 / sample_concentrations[i]),
                ABC,
                temp_plate.wells()[i * replicates: i * replicates + replicates],
                new_tip='once',
                touch_tip=True
            )
        else:
            p50.transfer(
                100 - (100 / sample_concentrations[i]),
```

```
            ABC,
            temp_plate.wells()[i * replicates: i * replicates + replicates],
            new_tip='once',
            touch_tip=True
        )

    # transfer 100ug of protein and mix 3 times with 50 uL volume; change 50 to 20 if p20 will be used.
        if (100 / sample_concentrations[i]) > 50:
            p300.transfer(
            100 / sample_concentrations[i],
            samples[i],
            temp_plate.wells()[i * replicates: i * replicates + replicates],
            mix_after=(3, 50),
            new_tip='always',
            touch_tip=True,
            blow_out=True,
            blowout_location='destination well'
            )
        else:
            p50.transfer(
            100 / sample_concentrations[i],
            samples[i],
            temp_plate.wells()[i * replicates: i * replicates + replicates],
            mix_after=(3, 50),
            new_tip='always',
            touch_tip=True,
            blow_out=True,
            blowout_location='destination well'
        )



  # | --------- transfer DTT to plate --------- |
  # change the change the mix volume from 50 to 20 if p20 will be used.
  protocol.pause('Ensure DTT has been loaded into A6 of the 2ml tube rack located in slot 4 prior to resuming protocol.')
  p50.transfer(
    volume_of_DTT,
    DTT,
    temp_plate.wells()[:number_of_samples * replicates],
    mix_after=(5, 50),
    new_tip='always',
    touch_tip=True,
```

```
        blow_out=True,
        blowout_location='destination well'
    )
    protocol.pause('Ensure to close caps on sample tubes.')

    # | --------- first incubation --------- |
    temp_mod.set_temperature(55)
    protocol.delay(minutes=5, msg='Pausing for 5 minutes to allow samples to reach tempeature.')
    protocol.delay(minutes=incubation_time_DTT, msg=f'Incubating at 55 degrees for {incubation_time_DTT} minutes.')

    # | --------- set block to room temp before adding IAA --------- |
    protocol.comment('Cooling down temp block.')
    temp_mod.set_temperature(22)
    protocol.delay(minutes=5, msg='Pausing for 5 minutes to allow tubes to cool down.')
    protocol.pause('Ensure to open caps on sample tubes.')

    # | --------- transfer IAA to samples on plate --------- |
    protocol.pause('Ensure IAA has been loaded into B6 of the 2ml tube rack located in slot 4 prior to resuming protocol.')
     # change the change the mix volume from 50 to 20 if p20 will be used.
    p50.transfer(
        volume_of_IAA,
        IAA,
        temp_plate.wells()[:number_of_samples * replicates],
        mix_after=(5, 50),
        new_tip='always',
        touch_tip=True,
        blow_out=True,
        blowout_location='destination well'
    )
    protocol.pause('Close caps on sample tubes and cover tubes with foil')

    # | --------- second incubation --------- |
    temp_mod.set_temperature(22)
    protocol.delay(minutes=incubation_time_IAA, msg=f'Protect tubes from light. Incubating at 22 degrees for {incubation_time_IAA} minutes.')
    protocol.comment('Temp block will now be deactivated.')
    temp_mod.deactivate()


    # | --------- transfer trypsin to samples on plate --------- |
    # change the change the mix volume from 50 to 20 if p20 will be used.
```

```
    protocol.pause('Ensure trypsin has been loaded into C6 of the 2ml tube rack located in slot 4 prior to resuming protocol.')
    protocol.pause('Open caps on sample tubes on the temperature module')
    p50.transfer(
        volume_of_trypsin,
        trypsin,
        temp_plate.wells()[:number_of_samples * replicates],
        mix_after=(5, 50),
        new_tip='always',
        touch_tip=True,
        blow_out=True,
        blowout_location='destination well')
    protocol.comment('Transfer to tubes to shaker for overnight digestion.')
```

SP3_peptide_cleanup.py

```
from opentrons import protocol_api

metadata = {
    'protocolName': 'SP3 Peptide Cleanup',
    'author': 'Cody',
    'description': 'Protocol for peptide cleanup using SP3 magnetic beads',
    'apiLevel': '2.8'
}


def run(protocol: protocol_api.ProtocolContext):
    # ---------------------------- CUSTOMIZE HERE ONLY ---------------------------- |
    number_of_samples: int = 3   # specify the number of protein digest
    replicates: int = 2        # specify the number of replicates per sample
    transfer_vol_peptides: float = 55.0 # specify the volume of digest to be processed; Each protein digest is about 120 uL and we split it into 2 cleanup reactions with each cleanup starting with 55 uL.
    volume_of_beads: float = 10.0  # Manually prepare beads for peptide binding prior to loading
    volume_of_ACN: float = 1292.0  # Volume of 100% ACN to be used during peptide binding phase; cannot exceed 1500uL
    volume_of_DMSO: float = 80.0  # Manually prepare 2% DMSO in MS water.
    total_samples = number_of_samples * replicates  # Total number of samples (including replicates) cannot exceed 48
    starting_tip_p50 = 'A1'  # change if full tip rack will not be used
    starting_tip_p300 = 'A1'  # change if full tip rack will not be used
    starting_mag_well = 0  # 0 corresponds to 'A1' up to 95 corresponding to 'H12'

    # | ---------------------------- ^^^^^^^^^^^^^^^^^^^^ ---------------------------- |
    # ---------------------------- DO NOT EDIT BELOW THIS LINE ---------------------------- #
```

```
    # | ---------  tip racks --------- |
    tiprack_300 = protocol.load_labware('opentrons_96_tiprack_300ul', 2)
    tiprack_300_2 = protocol.load_labware('opentrons_96_tiprack_300ul', 3)
    tiprack_50 = protocol.load_labware('opentrons_96_tiprack_300ul', 1)
    #tiprack_50_2 = protocol.load_labware('opentrons_96_tiprack_300ul', 6)

    # | ---------  pipettes --------- |
    p300 = protocol.load_instrument('p300_single', 'right', tip_racks=[tiprack_300, tiprack_300_2])
    p50 = protocol.load_instrument('p50_single', 'left', tip_racks=[tiprack_50]) #change p50 to p20 if p20 will be used here and throughout the script following OT-2 API; this script has not been tested with p20 therefore testing is required.
    p50.starting_tip = tiprack_50.well(starting_tip_p50)
    p300.starting_tip = tiprack_300.well(starting_tip_p300)
    p300_aspirate_slow = 25  # Aspiration speed when removing supernatant
    p300_aspirate_default = 150  # Normal aspiration speed by default
    p300_aspirate_fast = 200
    p50_aspirate_slow = 25  # Aspiration speed when removing supernatant;
    p50_aspirate_default = 150  # Normal aspiration speed by default;

    # | ---------  tube racks/plates/containers --------- |
    mag_deck = protocol.load_module('magdeck', 7)
    if mag_deck.status == 'engaged':
        mag_deck.disengage()

    mag_plate = mag_deck.load_labware('nest_96_wellplate_2ml_deep')
    tuberack_2mL = protocol.load_labware('opentrons_24_tuberack_nest_2ml_snapcap', 4)
    tuberack_15ml_50ml = protocol.load_labware('opentrons_10_tuberack_falcon_4x50ml_6x15ml_conical', 5)

    # | --------- reagents --------- |
    beads = tuberack_2mL['A6']
    DMSO = tuberack_15ml_50ml['A1']
    ACN = tuberack_15ml_50ml['A3']
    waste = tuberack_15ml_50ml['B3']
    samples = tuberack_2mL.wells()[:number_of_samples]

    # ---------------------------- COMMANDS ---------------------------- #

    # Check total number of samples and replicates
    if (starting_mag_well + total_samples * 2 > 96):
        raise Exception("Well plate does not have the required number of wells to hold all replicates at that starting position.")
```

```
    # Function for resuspending beads in a given volume of a specified reagent
    def reagentTransfer(vol, reagent, wells=mag_plate.wells()[starting_mag_well: total_samples +
starting_mag_well]):
        for well in wells:
            p300.pick_up_tip()
            p300.transfer(
                vol,
                reagent,
                well.top() if reagent == ACN else well,
                mix_before=(3, 100) if reagent == DMSO else None,
                air_gap=10,
                touch_tip=True if reagent == DMSO else False,
                new_tip='never',
                blow_out=True,
                blowout_location='destination well',
            )
            p300.mix(10, vol if vol < 300 else 300, well.bottom(1))
            p300.touch_tip()
            p300.blow_out()
            p300.drop_tip()

    # Function for mixing resuspended beads to mimic mixing on a plate shaker
    def  mixWells(mix_vol,  num_mixes,  delay_min,  wells=mag_plate.wells()[starting_mag_well:
total_samples + starting_mag_well]):
        curr_mix = 0
        while curr_mix < num_mixes:
            protocol.delay(minutes=delay_min)
            for well in wells:
                p300.pick_up_tip()
                p300.mix(10, mix_vol if mix_vol < 300 else 300, well.bottom(1))
                p300.blow_out()
                p300.touch_tip()
                p300.drop_tip()
            curr_mix += 1


     Transfer defined mass of peptide from sample to the plate on magnetic module

     for i in range(len(samples)):
        p300.flow_rate.aspirate = p300_aspirate_slow
        p300.flow_rate.dispense = p300_aspirate_slow
        p300.transfer(
        transfer_vol_peptides,
        samples[i],
```

```
        mag_plate.wells()[i * replicates + starting_mag_well: i * replicates + replicates + starting_mag_well],
        touch_tip=True,
        new_tip='once',
        blow_out=True,
        blowout_location='destination well'
        )

    p300.flow_rate.aspirate = p300_aspirate_default
    p300.flow_rate.dispense = p300_aspirate_default

    # Transfer beads, then ACN to the tubes with peptide samples
    protocol.pause('Ensure prepared beads have been loaded into A6 of the 2ml tube rack located in slot 4 prior to resuming protocol.')
    p50.flow_rate.aspirate = p50_aspirate_default
    p50.flow_rate.dispense = p50_aspirate_default

    #change p50 to p20 if needed
    p50.transfer(
        volume_of_beads,
        beads,
        mag_plate.wells()[starting_mag_well:total_samples + starting_mag_well],
        mix_before=(5, 50),
        mix_after=(5, 50),
        new_tip='always',
        touch_tip=True,
        blow_out=True,
        blowout_location='destination well'
    )


    reagentTransfer(volume_of_ACN, ACN)
    mixWells(mix_vol=volume_of_ACN, num_mixes=5, delay_min=0.0)
    mag_deck.engage()
    protocol.delay(minutes=2, msg='Incubating on magnet for 2 minutes.')

    # Remove supernatant after initial incubation
    # Reduce aspiration speed prior to removing supernatant
    p300.flow_rate.aspirate = p300_aspirate_slow
    p300.flow_rate.dispense = p300_aspirate_default
    for mag_well in mag_plate.wells()[starting_mag_well:total_samples + starting_mag_well]:
        p300.pick_up_tip()
        p300.transfer(
            volume_of_ACN * 1.1,
```

```
        mag_well.bottom(1),
        waste.top(),
        air_gap=10,
        new_tip='never'
    )
    p300.touch_tip()
    p300.blow_out(waste)
    p300.drop_tip()
# Return aspiration speed back to default before moving on in the protocol execution
p300.flow_rate.aspirate = p300_aspirate_default
mag_deck.disengage()

# # Wash beads with 1mL ACN
protocol.pause('make sure ACN tube caps are off')
reagentTransfer(1000, ACN)
mixWells(mix_vol=1000, num_mixes=1, delay_min=0)
mag_deck.engage()
protocol.delay(minutes=2, msg='Incubating on magnet for 2 minutes.')

# Remove supernatant after wash incubation
# Reduce aspiration speed prior to removing supernatant
p300.flow_rate.aspirate = p300_aspirate_slow
for mag_well in mag_plate.wells()[starting_mag_well:total_samples+starting_mag_well]:
    p300.pick_up_tip()
    p300.transfer(
        1000 * 1.1,
        mag_well.bottom(1),
        waste.top(),
        air_gap=10,
        new_tip='never'
    )
    p300.blow_out(waste)
    p300.drop_tip()
# Return aspiration speed back to default before moving on in the protocol execution
p300.flow_rate.aspirate = p300_aspirate_default
protocol.delay(seconds=60, msg='Delaying for 60 seconds to allow residual ACN to evaporate.')
mag_deck.disengage()

# # Peptide elution
# Transfer 2% DMSO to samples
protocol.pause('vortex DMSO again and open caps.')
reagentTransfer(volume_of_DMSO, DMSO)
mixWells(mix_vol=volume_of_DMSO, num_mixes=4, delay_min=0)
mag_deck.engage()
```

```
    protocol.delay(minutes=2, msg='Incubating on magnet for 2 minutes.')

    # # Transfer first elution volumes to empty wells on the plate
    # # Reduce aspiration speed prior to removing supernatant
    p300.flow_rate.aspirate = p300_aspirate_slow
    for mag_well, dest_well in zip(mag_plate.wells()[starting_mag_well:total_samples + starting_mag_well],
                                   mag_plate.wells()[total_samples + starting_mag_well:total_samples * 2 + starting_mag_well]):
        p300.pick_up_tip()
        p300.transfer(
            volume_of_DMSO * 1.2,
            mag_well.bottom(0.5),
            dest_well,
            new_tip='never',
            blow_out=True,
            blowout_location='destination well'
        )
        p300.drop_tip()
    protocol.delay(minutes=2, msg='Incubating on magnet for 2 minutes to remove any residual beads in solution.')

    # # Transfer final elution volumes to new tubes on the 2mL tube rack
    # protocol.pause('Ensure enough 2mL LoBind tubes are in the 2mL tube rack to match total number of samples')
    for mag_well, dest_well in zip(mag_plate.wells()[total_samples + starting_mag_well: total_samples*2 + starting_mag_well],
                                   tuberack_2mL.wells()[number_of_samples:number_of_samples + total_samples]):
        p300.pick_up_tip()
        p300.transfer(
            volume_of_DMSO * 1.1,
            mag_well,
            dest_well,
            new_tip='never',
            blow_out=True,
            blowout_location='destination well'
        )
        p300.drop_tip()

    # Return aspiration speed back to default before moving on in the protocol execution
    p300.flow_rate.aspirate = p300_aspirate_default
    mag_deck.disengage()
```

```
    # Final check to disengage magnetic module if it hasn't disengaged
    if mag_deck.status == 'engaged':
        mag_deck.disengage()
```

SP3_digestion.py

```
from opentrons import protocol_api


metadata = {
    'protocolName': 'SP3 Protein Cleanup and Digestion',
    'author': 'Cody',
    'description': 'Digestion protocol with SP3 detergent removal',
    'apiLevel': '2.8'
}


def run(protocol: protocol_api.ProtocolContext):
    # ---------------------------- CUSTOMIZE HERE ONLY ---------------------------- |
    number_of_samples: int = 1 # Specify the number of protein samples

    sample_concentrations = [5.00]  # specify the concentration of protein samples (unit is ug/uL); Length of sample_concentrations list must match the number of the samples above; separate concentrations with period sign if sample n>1 (e.g. sample_concentrations=[2.0, 2.5] if sample n=2).
    replicates: int = 3 # specify the number of replicates for each sample
    volume_of_DTT: float = 10.0  # manually prepare 60mM DTT in MS-grade water
    volume_of_IAA: float = 10.0  # manually prepare 375mM IAA in MS-grade water
    volume_of_trypsin: float = 10.0  # manually prepare to a concentration of 0.2ug/uL
    incubation_time_DTT = 30  # in minutes
    incubation_time_IAA = 30  # in minutes
    volume_of_beads: float = 20.0  # Manually prepare beads for peptide binding prior to loading
    volume_of_ethanol100: float = 140.0  # Volume of 100% ethanol to be used during protein binding phase
    volume_of_ethanol80: float = 1000.0  # Volume of 80% ethanol to be used for washes
    total_samples = number_of_samples * replicates  # Total number of samples (including replicates) cannot exceed 24
    starting_tip_p50 = 'A1'  # change if full tip rack will not be used
    starting_tip_p300 = 'A1'  # change if full tip rack will not be used
    starting_mag_well = 0  # 0 corresponds to 'A1' up to 95 corresponding to 'H12'

    # | ---------  tip racks --------- |
    tiprack_300 = protocol.load_labware('opentrons_96_tiprack_300ul', 3)
    tiprack_300_2 = protocol.load_labware('opentrons_96_tiprack_300ul', 6)
```

```
    tiprack_50 = protocol.load_labware('opentrons_96_tiprack_300ul', 1)
    tiprack_50_2 = protocol.load_labware('opentrons_96_tiprack_300ul', 2)

    # | ---------  pipettes --------- |
    #p300 = protocol.load_instrument('p300_single', 'right', tip_racks=[tiprack_300])
    p300 = protocol.load_instrument('p300_single', 'right', tip_racks=[tiprack_300, tiprack_300_2])
    p50 = protocol.load_instrument('p50_single', 'left', tip_racks=[tiprack_50, tiprack_50_2]) #change p50 to p20 if p20 will be used here and throughout the script following OT-2 API; this script has not been tested with p20 therefore testing is required"
    p50.starting_tip = tiprack_50.well(starting_tip_p50)
    p300.starting_tip = tiprack_300.well(starting_tip_p300)
    p300_aspirate_slow = 25  # Aspiration speed when removing supernatant
    p300_aspirate_default = 150  # Normal aspiration speed by default


    # | ---------  tube racks/plates/containers --------- |
    temp_mod = protocol.load_module('Temperature Module', 10)
    temp_plate = temp_mod.load_labware('opentrons_24_aluminumblock_nest_2ml_snapcap')
    tuberack_2mL = protocol.load_labware('opentrons_24_tuberack_nest_2ml_snapcap', 4)
    tuberack_15ml_50ml = protocol.load_labware('opentrons_10_tuberack_falcon_4x50ml_6x15ml_conical', 5)
    mag_deck = protocol.load_module('magdeck', 7)
    if mag_deck.status == 'engaged':
        mag_deck.disengage()
    mag_plate = mag_deck.load_labware('nest_96_wellplate_2ml_deep')

    # | --------- reagents --------- |
    samples = tuberack_2mL.wells()[:number_of_samples]
    DTT = tuberack_2mL['A6']
    IAA = tuberack_2mL['B6']
    trypsin = tuberack_2mL['C6']
    beads = tuberack_2mL['D6']
    ABC = tuberack_15ml_50ml['A1']
    ethanol100 = tuberack_15ml_50ml['A3']
    ethanol80 = tuberack_15ml_50ml['A4']
    waste = tuberack_15ml_50ml['B3']

    # ---------------------------- COMMANDS ---------------------------- #
    # Check well plate for adequate number of wells available after the starting well
    if (starting_mag_well + total_samples > 95):
        raise Exception("Well plate does not have the required number of wells to hold all replicates at that starting position.")
```

```
    # Function for resuspending beads in a given volume of a specified reagent
    def reagentTransfer(vol, reagent, wells=mag_plate.wells()[starting_mag_well: total_samples +
starting_mag_well]):
        for well in wells:
            p300.pick_up_tip()
            p300.transfer(
                vol,
                reagent,
                well.top() if reagent == ethanol80 else well,
                air_gap=10,
                new_tip='never',
                blow_out=True,
                blowout_location='destination well',
            )
            p300.mix(10, vol if vol < 300 else 300, well.bottom(1))
            p300.blow_out()
            p300.drop_tip()

    #  Function for mixing resuspended beads to mimic mixing on a plate shaker
    def  mixWells(mix_vol,  num_mixes,  delay_min,  wells=mag_plate.wells()[starting_mag_well:
total_samples + starting_mag_well]):
        curr_mix = 0
        while curr_mix < num_mixes:
            protocol.delay(minutes=delay_min)
            for well in wells:
                p300.pick_up_tip()
                p300.mix(5, mix_vol if mix_vol < 300 else 300, well.bottom(1))
                p300.touch_tip()
                p300.blow_out()
                p300.drop_tip()
            curr_mix += 1

    # Transfer 100mM ABC then 100ug of protein from samples to tubes on temp plate.
Concentration in tubes will be 1 ug/uL
    mass_of_protein = 100.0
    for i in range(number_of_samples):
        # transfer ABC; change 50 to 20 if p20 will be used
        if (mass_of_protein - (mass_of_protein / sample_concentrations[i])) > 50:
            p300.transfer(
                100 - (mass_of_protein / sample_concentrations[i]),
                ABC,
                temp_plate.wells()[i * replicates: i * replicates + replicates],
                new_tip='once',
                touch_tip=True,
```

```
            blow_out=True,
            blowout_location='destination well'
        )
    else:
        p50.transfer(
            100 - (mass_of_protein / sample_concentrations[i]),
            ABC,
            temp_plate.wells()[i * replicates: i * replicates + replicates],
            new_tip='once',
            touch_tip=True,
            blow_out=True,
            blowout_location='destination well'
        )


    # transfer 100ug of protein and mix 3 times with 50 uL volume; change 50 to 20 if p20 will be used
    if (mass_of_protein / sample_concentrations[i]) > 50:
        p300.transfer(
        mass_of_protein / sample_concentrations[i],
        samples[i],
        temp_plate.wells()[i * replicates: i * replicates + replicates],
        mix_after=(3, 50),
        new_tip='always',
        touch_tip=True,
        blow_out=True,
        blowout_location='destination well'
        )
    else:
        p50.transfer(
        mass_of_protein / sample_concentrations[i],
        samples[i],
        temp_plate.wells()[i * replicates: i * replicates + replicates],
        mix_after=(3, 50),
        new_tip='always',
        touch_tip=True,
        blow_out=True,
        blowout_location='destination well'
        )

  # transfer DTT to tubes on temp plate and change the mix volume from 50 to 20 if p20 will be used.
  protocol.pause('Ensure DTT has been loaded into A6 of the 2ml tube rack located in slot 4 prior to resuming protocol.')
```

```
    p50.transfer(
        volume_of_DTT,
        DTT,
        temp_plate.wells()[:number_of_samples * replicates],
        mix_after=(5, 50),
        new_tip='always',
        touch_tip=True,
        blow_out=True,
        blowout_location='destination well'
    )
    protocol.pause('Ensure to close caps on sample tubes.')

    # DTT incubation
    temp_mod.set_temperature(55)
    protocol.delay(minutes=5, msg='Pausing for 5 minutes to allow samples to reach tempeature.')
    protocol.delay(minutes=incubation_time_DTT, msg=f'Incubating at 55 degrees for {incubation_time_DTT} minutes.')

    # cool temp block and tubes to room temp prior to adding IAA to samples
    protocol.comment('Cooling down temp block.')
    temp_mod.set_temperature(22)
    protocol.delay(minutes=5, msg='Pausing for 5 minutes to allow tubes to cool down.')
    protocol.pause('Ensure to open caps on sample tubes.')

    # transfer IAA to tubes on temp plate and change the mix volume from 50 to 20 if p20 will be used.
    protocol.pause('Ensure IAA has been loaded into B6 of the 2ml tube rack located in slot 4 prior to resuming protocol.')
    p50.transfer(
        volume_of_IAA,
        IAA,
        temp_plate.wells()[:number_of_samples * replicates],
        mix_after=(5, 50),
        new_tip='always',
        touch_tip=True,
        blow_out=True,
        blowout_location='destination well'
    )
    protocol.pause('Close caps on sample tubes and cover tubes with foil')

    # IAA incubation
    temp_mod.set_temperature(22)
    protocol.delay(minutes=incubation_time_IAA,
```

```
            msg=f'Protect tubes from light. Incubating at 22 degrees for {incubation_time_IAA}
minutes.')
   protocol.comment('Temp block will now be deactivated.')
   temp_mod.deactivate()
   protocol.pause('open tube caps')


   #Transfer protein samples from tubes to the deep-well plate on magnetic module
   for i in range(total_samples):
      p300.transfer(
         120 * 1.1,
         temp_plate.wells()[i * replicates: i * replicates + replicates],

         mag_plate.wells()[(starting_mag_well + i * replicates) : (starting_mag_well + i * replicates
+ replicates)],
         new_tip='always',
         touch_tip=True,
         blow_out=True,
         blowout_location='destination well'
      )

   # add beads to samples
   protocol.pause('Ensure prepared beads have been loaded into D6 of the 2ml tube rack located
in slot 4 prior to resuming protocol.')
   p50.transfer(
      volume_of_beads,
      beads,
      mag_plate.wells()[starting_mag_well: total_samples + starting_mag_well],
      mix_before=(5, volume_of_beads),
      mix_after=(5, volume_of_beads),
      new_tip='always',
      blow_out=True,
      blowout_location='destination well'
   )

   protocol.pause('Ensure 100 percent ethanol has been loaded into A3 of the 15mL_50mL tube
rack located in slot 5 prior to resuming protocol.')
   reagentTransfer(volume_of_ethanol100, ethanol100)
   mixWells(mix_vol=volume_of_ethanol100, num_mixes=5, delay_min=0)
   mag_deck.engage()
   protocol.delay(minutes=2, msg='Incubating on magnet for 2 minutes.')

   # Remove supernatant after initial incubation
   # Reduce aspiration speed prior to removing supernatant
```

```
    p300.flow_rate.aspirate = p300_aspirate_slow
    # for mag_well in mag_plate.wells()[:total_samples]:
    for mag_well in mag_plate.wells()[starting_mag_well: total_samples + starting_mag_well]:
        p300.pick_up_tip()
        p300.transfer(
            volume_of_ethanol100 * 1.1,
            mag_well.bottom(1),
            waste.top(),
            air_gap=10,
            new_tip='never'
        )
        p300.touch_tip()
        p300.blow_out(waste)
        p300.drop_tip()
    # Return aspiration speed back to default before moving on in the protocol execution
    p300.flow_rate.aspirate = p300_aspirate_default
    mag_deck.disengage()

    # Wash beads with 80% ethanol (3 washes in total)
    protocol.pause('Ensure 80 percent ethanol has been loaded into A4 of the 15mL_50mL tube
rack located in slot 5 prior to resuming protocol.')
    for i in range(3):
        if mag_deck.status == 'engaged':
            mag_deck.disengage()
        reagentTransfer(volume_of_ethanol80, ethanol80)
        mag_deck.engage()
        protocol.delay(minutes=2, msg='Incubating on magnet for 2 minutes.')

        # Remove supernatant after wash incubation
        # Reduce aspiration speed prior to removing supernatant
        p300.flow_rate.aspirate = p300_aspirate_slow
        # for mag_well in mag_plate.wells()[:total_samples]:
        for mag_well in mag_plate.wells()[starting_mag_well: total_samples + starting_mag_well]:
            p300.pick_up_tip()
            p300.transfer(
                volume_of_ethanol80 * 1.1,
                mag_well.bottom(1),
                waste.top(),
                air_gap=10,
                new_tip='never'
            )
            p300.blow_out(waste)
            p300.drop_tip()
        p300.flow_rate.aspirate = p300_aspirate_default
```

```
    # Wash beads with 250 uL ABC
    protocol.pause('Open cap on ABC tube.')
    if mag_deck.status == 'engaged':
        mag_deck.disengage()
    reagentTransfer(250, ABC)
    mixWells(mix_vol=250, num_mixes=0, delay_min=0)
    mag_deck.engage()
    protocol.delay(minutes=2, msg='Incubating on magnet for 2 minutes.')

    # Remove supernatant after wash incubation
    # Reduce aspiration speed prior to removing supernatant
    p300.flow_rate.aspirate = p300_aspirate_slow
    # for mag_well in mag_plate.wells()[:total_samples]:
    for mag_well in mag_plate.wells()[starting_mag_well: total_samples + starting_mag_well]:
        p300.pick_up_tip()
        p300.transfer(
            250 * 1.1,
            mag_well.bottom(1),
            waste.top(),
            air_gap=10,
            new_tip='never'
        )
        p300.blow_out(waste)
        p300.drop_tip()
    # Return aspiration speed back to default before moving on in the protocol execution
    p300.flow_rate.aspirate = p300_aspirate_default

    mag_deck.disengage()

    # resuspend proteins and beads in 100uL of 100mM ABC and move to 2mL tubes for incubation
    reagentTransfer(100, ABC)
    protocol.pause('Ensure new collection tubes have been placed in 2.0 mL aluminum block prior
to resuming protocol.')
    p300.transfer(
        100 * 1.5,
        # mag_plate.wells()[:total_samples],
        mag_plate.wells()[starting_mag_well: total_samples + starting_mag_well],
        temp_plate.wells()[number_of_samples:number_of_samples + total_samples],
        mix_before=(10, 100),
        new_tip='always',
        touch_tip=True,
```

```
        blow_out=True,
        blow_out_location='destination well'
    )

    # transfer trypsin to each sample and change the mix volume from 50 to 20 if p20 will be used
    protocol.pause('Ensure trypsin (0.2ug/uL) has been loaded into C6 of the 2ml tube rack located
in slot 4 prior to resuming protocol.')
    p50.transfer(
        volume_of_trypsin,
        trypsin,
        temp_plate.wells()[:total_samples],
        mix_after=(5, 50),
        new_tip='always',
        touch_tip=True,
        blow_out=True,
        blowout_location='destination well')
    protocol.comment('Transfer digest tubes to plate shaker for overnight digestion.')
```

Supplementary File 2

The following method parameters pertain to Protocol Step 6.3:

Peptide digests are separated at 300 nL/min with a 120 min gradient for the heart sample: 0 - 105 min: 0 to 40% B; 105 - 110 min: 40 to 70% B; 110 - 115 min: 70 to 100% B; 115 - 120 min: 100% B (solvent A: 0.1% v/v formic acid in water; solvent B: 0.1% v/v formic acid in 80% acetonitrile; column temperature: 50 °C). For the BSA sample, a 20 min gradient was used: 0 -10 min: 0 to 30% B; 10 - 15 min: 30 to 80% B; 15 - 20 min: 80 % B.

Supplementary File 3

The following method parameters pertain to Protocol Step 6.4:

In a typical setup, spectra may be acquired with the following method: polarity: positive; data dependent acquisition (DDA): top 10 ions (BSA samples) or 15 ions (heart samples); MS resolution: 60,000; mass range: 200-1650 m/z; precursor dynamic exclusion: 30s; maximum ion injection time: 20 ms; MS automatic gain control (AGC) target: 3e6; isolation window: 2.0 m/z; stepped normalized collision energy (NCE): 25, 27, 29; MS2 resolution; 60,000; MS2 maximum ion injection time: 110 ms; MS2 AGC target: 2e5. Customize this step based on the instrumentation platform