Automated Workflow for Protein Digestion, Tandem Mass Tag Labeling and Clean-up for Multiplex LC-MS Analysis

Written by
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Introduction

Protein digestion and clean-up are critical steps in preparing high-quality peptides for mass spectrometry (MS)-based proteomic studies. The process is time- and labor-intensive. To enable simultaneous analysis across multiple samples, increase throughput and reduce run-to-run variability, tandem mass tags (TMT) can be used for sample multiplexing, adding even further complex bench work.

Lab automation for peptide sample preparation can significantly reduce hands-on time, minimize the risk of human error, and increase throughput. Here, we present a fully automated workflow on the Opentrons Flex® for trypsin/Lys-C-mediated protein digestion, magnetic bead-based clean-up, and TMT labeling using commercially available kits.

Methods

1. Samples (purified BSA or HeLa whole cell lysate), reagents (EasyPep Magnetic MS Sample Prep Kit and TMT Mass Tagging Kit, Thermo Fisher Scientific, Waltham, MA, USA) and supporting labware were loaded manually onto the Opentrons Flex® liquid handling robot.

2. Two protocols were developed and optimized on the Opentrons Flex®, equipped with a fully integrated thermocycler to provide precise temperature control.
   - Protocol #1: Protein Digestion and TMT labeling (Figure 1)
   - Protocol #2: Sample Clean-up (Figure 2)

3. The quality of final products was assessed by LC-MS analysis.

Sample Prep for MS (EasyPep Digestion and TMT Labeling)

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This protocol performs protein digestion by using EasyPep Magnetic MS Sample Preparation Kit (Thermo Fisher Scientific, Waltham, MA, USA) and peptides labeled by using TMT Mass Tagging Kit (Thermo Fisher Scientific, Waltham, MA, USA), for proteomic MS analysis, followed by sample clean-up: **Sample Prep for MS (EasyPep Cleanup)**

https://library.opentrons.com/p/easypep_digest_tmt

Sample Prep for MS (EasyPep Cleanup)

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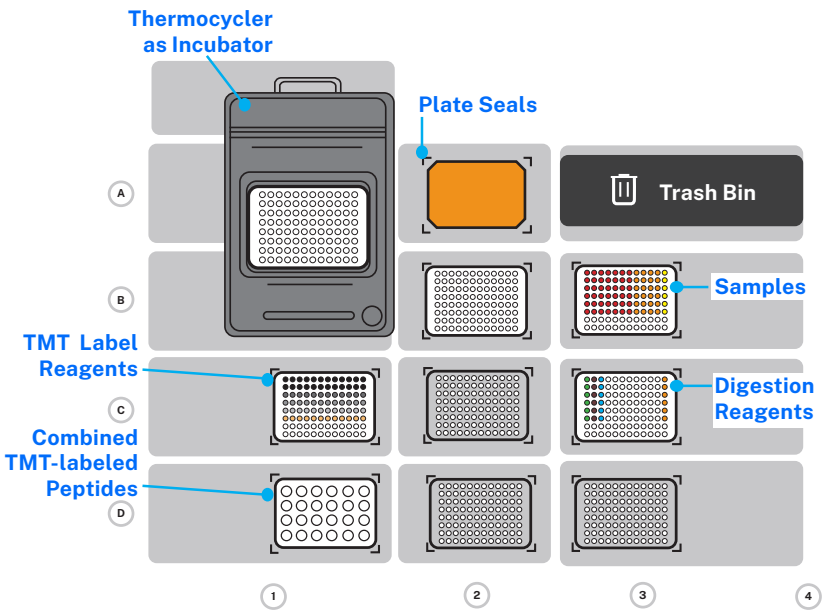
This protocol performs sample clean-up by using EasyPep Magnetic MS Sample Preparation Kit (Thermo Fisher Scientific, Waltham, MA, USA) for proteomic MS analysis, following protein digestion and TMT labeling: **Sample Prep for MS (EasyPep Digestion and TMT Labeling)**

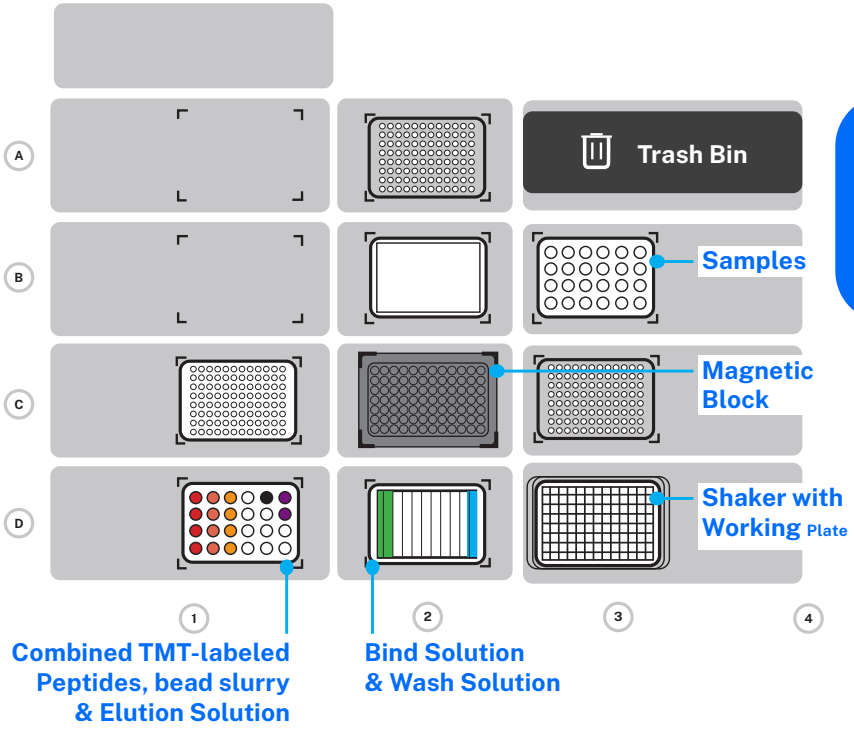
https://library.opentrons.com/p/easypep_cleanup



Protein Digestion
- Transfer reduction solution
- Transfer alkylation solution
- Incubate at 50° C
- Transfer enzyme solution
- Incubate at 37° C

TMT labeling
- Transfer TMT reagent
- Incubate at room temperature
- Transfer 5% hydroxylamine
- Incubate at room temperature

Figure 1. Protocol #1: Protein Digestion and TMT labeling. The Flex deck layout (left) and workflow (right).



Sample Clean-up
- Wash Beads
- Bind Peptides
- Wash Peptides
- Elute Peptides

Figure 2. Protocol #2: Sample Clean-up. The Flex deck layout (left) and the workflow (right).

Results

Protocol validation using BSA (Figure 3)

1. For each sample, purified BSA was digested, labeled with TMT2-126 or TMT2-127 isobaric reagent, combined in a 1:1 ratio, and desalted prior to LC-MS analysis.

2. Across three independent tests processed using the Opentrons Flex, data indicate consistently equal TMT labeling (average 127/126 ratio of 1.039), which is comparable to the standard manual procedure.

	127/126 ratio
Flex #1	0.926
Flex #2	1.045
Flex #3	1.145
Manual	0.98

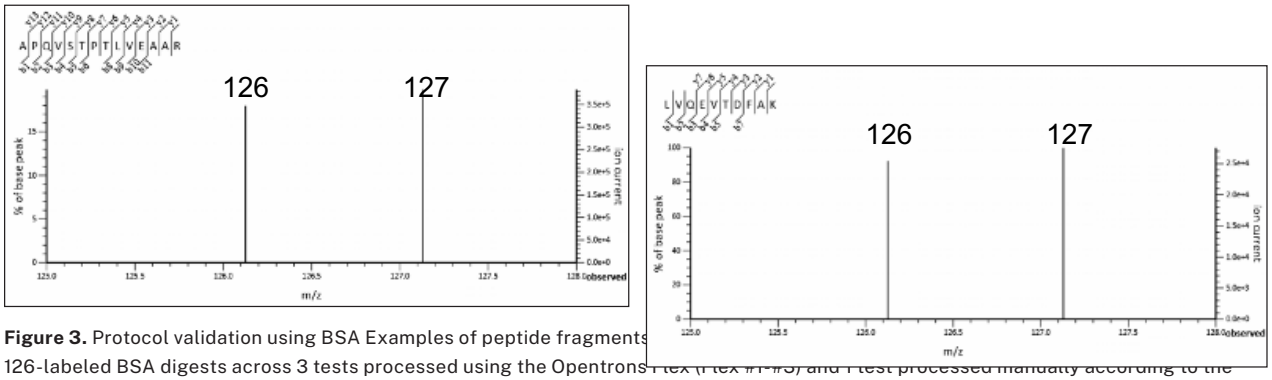


Figure 3. Protocol validation using BSA Examples of peptide fragments 126-labeled BSA digests across 3 tests processed using the Opentrons Flex (Flex #1-#3) and 1 test processed manually according to the vendor's instructions (upper)

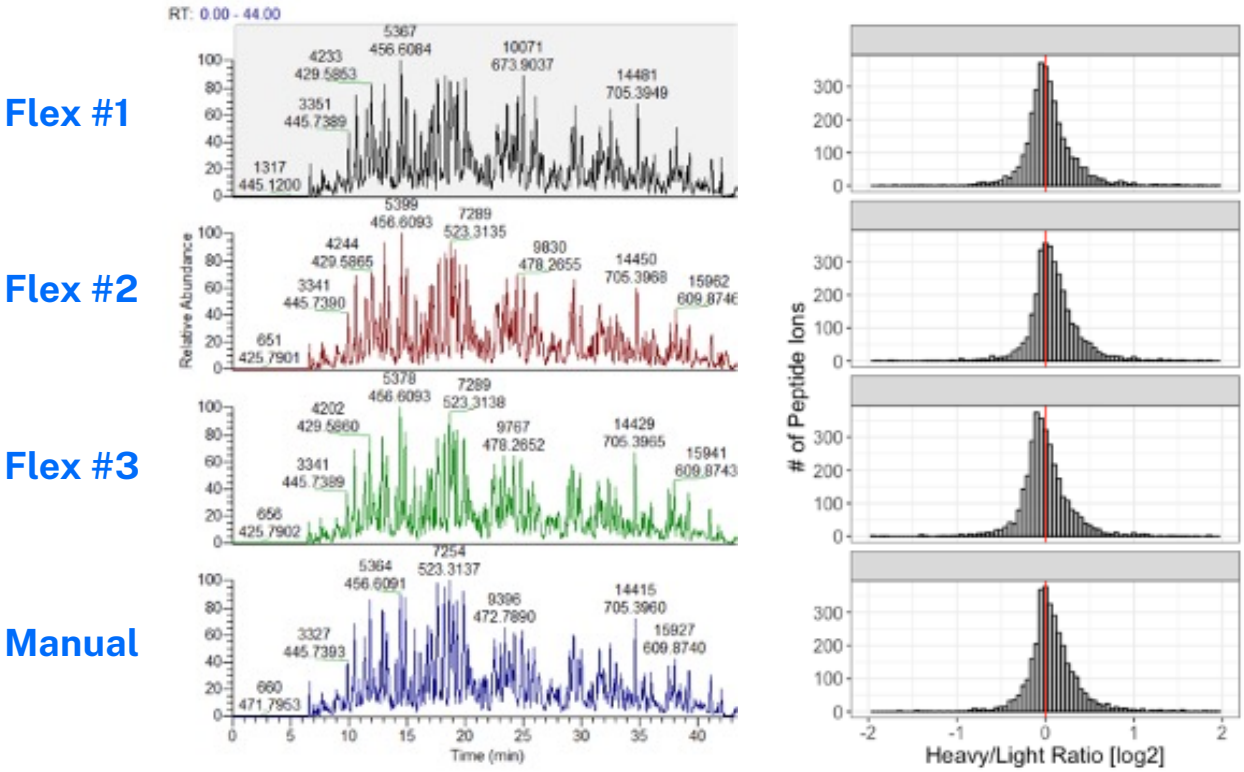
Results

Sample preparation for HeLa whole cell lysate (Figure 4)

1. For each sample, two batches of HeLa cell lysate (100 µg) were digested, labeled with TMT6-127 and TMTzero isotopic tags, respectively, combined in a 1:1 ratio, and desalted prior to LC-MS analysis.

2. Digested peptides were fully labeled (99% labeling efficiency) with two isotopic TMTs at approximately equal ratio (heavy/light ratio ~1).

3. Well-aligned base peak chromatograms and other data, including labeling efficiency for sample multiplexing were consistent across 3 independent tests, where samples were processed using the Opentrons Flex. Data were comparable to the manual procedure, highlighting the accuracy of the automation workflow



		Precursor Count			
		Flex #1	Flex #2	Flex #3	Manual
TMT6-127	Fully-labeled	3009	2932	3111	3032
	Semi-labeled	25	31	27	29
	Unlabeled	0	0	0	0
	Labeling Efficiency	99%	99%	99%	99%
TMTzero	Fully-labeled	3086	3218	3124	3214
	Semi-labeled	21	20	17	21
	Unlabeled	10	5	6	7
	Labeling Efficiency	99%	99%	99%	99%

Figure 4. Sample preparation for HeLa whole cell lysate. Base peak chromatogram (upper left), ratio of two TMT-labeled peptides (TMT6-127: Heavy; TMTzero-126: Light) (upper right), and labeling efficiency (lower) of 3 tests processed using the Opentrons Flex (Flex #1-#3) and 1 test processed manually according to the vendor's instructions.

Conclusion

A complete peptide preparation workflow on the Opentrons Flex® was developed to support full automation of TMT-assisted multiplex LC-MS with desirable consistency, increased throughput, and quality of sample handling. Here, automation significantly reduced hands-on time to less than 10 minutes for reagent preparation and labware loading, saving valuable time at the bench.

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