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TITLE

Carrier-assisted One-pot Sample Preparation for Targeted Proteomics Analysis of Small Numbers of Human Cells

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SUMMARY:

A protein carrier-assisted one-pot sample preparation coupled with liquid chromatography (LC) – selected reaction monitoring (SRM) termed cLC-SRM is a convenient method for multiplexed targeted proteomics analysis of small numbers of cells, including single cells. It capitalizes on using excessive exogenous protein as a carrier and high-specificity LC-SRM for targeted quantification.

ABSTRACT:

Protein analysis of small numbers of human cells is primarily achieved by targeted proteomics with antibody-based immunoassays, which have inherent limitations (e.g., low multiplex and unavailability of antibodies for new proteins). Mass spectrometry (MS)-based targeted proteomics has emerged as an alternative because it is antibody-free, high multiplex, and has high specificity and quantitation accuracy. Recent advances in MS instrumentation make MS-based targeted proteomics possible for multiplexed quantification of highly abundant proteins in single cells. However, there is a technical challenge for effective processing of single cells with minimal sample loss for MS analysis. To address this issue, we have recently developed a convenient protein carrier-assisted one-pot sample preparation coupled with liquid chromatography (LC) – selected reaction monitoring (SRM) termed cLC-SRM for targeted proteomics analysis of small numbers of human cells. This method capitalizes on using the combined excessive exogenous protein as a carrier and low-volume one-pot processing to greatly reduce surface adsorption losses as well as high-specificity LC-SRM to effectively address the increased dynamic concentration range due to the addition of exogeneous carrier protein. Its utility has been demonstrated by accurate quantification of most moderately abundant proteins in small numbers of cells (e.g., 10-100 cells) and highly abundant proteins in single cells. The easy-to-implement features and no need for specific devices make this method readily accessible to most proteomics laboratories. Herein we have provided a detailed protocol for cLC-SRM analysis of small numbers of human cells including cell sorting, cell lysis and digestion, LC-SRM analysis, and data analysis. Further improvements in detection sensitivity and sample throughput are needed towards targeted single-cell proteomics analysis. We anticipate that cLC-SRM will be broadly applied to biomedical research and systems biology with the potential of facilitating precision medicine.

INTRODUCTION:

Recent technological advances in genomics (transcriptomics) allow for comprehensive and precise analysis of the genome (transcriptome) in single cells[1-3]. However, single-cell proteomics technologies are lagging far behind but are just as important as genomics (transcriptomics) technologies[4-8]. Furthermore, protein abundance cannot necessarily be inferred from mRNA abundance[9], and the proteome is more complex and dynamic than the transcriptome[10]. Given these challenges, a large number of mixed populations of cells (i.e., bulk cells) are generally used to generate comprehensive proteome data[11-13]. However, such bulk measurements average out stochastic variations of individual cells, thus obscure important cell-to-cell variability (i.e., cell heterogeneity)[4,14]. Limitations of such bulk measurements become even more severe when the cells of interest only account for a small portion of the total populations of cells (e.g., cancer stem cells within tumors at an early-stage cancer). Therefore, there is a huge knowledge gap between single-cell proteomics and genomics (transcriptomics).

Antibody-based immunoassays (e.g., flow or mass cytometry) are predominantly used for targeted proteomic analysis of single cells[6,7,15-18]. However, they suffer from low multiplex, limited specificity, and unavailability of antibodies for new proteins of interest. Mass spectrometry (MS)-based targeted proteomics has emerged as an alternative for accurate protein quantification because of its being antibody-free, high multiplex (≥150 proteins in a single

analysis[19]), high quantitation accuracy (absolute amounts or concentrations), and high specificity and reproducibility (≤10% CV)[20-23]. Recent significant progress in sample preparation has made MS-based single-cell proteomics possible for quantitative analysis of highly abundant proteins from single human cells. However, MS-based single-cell proteomics is still at the early infancy stage. For example, the most advanced MS platform coupled with ultralow-flow RPLC flow rates can only allow label-free MS detection and quantification of ~670-870 proteins out of the total ≥12,000 proteins in single HeLa cells[24,25].

Currently, there are six MS-based single-cell proteomics approaches available for analysis of single mammalian cells, in which four are for global proteomics (nanoPOTS: nanowell-based Preparation in One pot for Trace Samples[26]; iPAD-1: integrated proteome analysis device for single-cell analysis[27]; OAD (oil-air-droplet) chip-based single cell proteomic analysis[28]; SCoPE-MS: single cell proteomics by mass spectrometry[29]) and the other two are for targeted proteomics (cLC-SRM: carrier-assisted liquid chromatography (LC) – selected reaction monitoring (SRM)[30]; cPRISM-SRM: carrier-assisted high pressure, high-resolution separations with intelligent selection and multiplexing coupled to SRM[31]). However, all these approaches have technical drawbacks. nanoPOTS, iPAD-1, and OAD downscale sample processing volume to 2-200 nL and are not ready for broad benchtop applications[26-28]. For SCoPE-MS, a TMT (tandem mass tag) carrier is added after single-cell processing, so it cannot effectively prevent surface adsorption losses during sample processing when a single tube is used for single-cell processing[29], resulting in low reproducibility with a correlation coefficient of only ~0.2-0.4 between replicates[32]. For cLC-SRM and cPRISM-SRM, using exogenous proteins as a carrier is more suitable for targeted proteomics because peptides from excessive exogenous proteins are frequently sequenced by MS/MS, which greatly reduces the chance for sequencing low abundant endogenous peptides[30,31]. Unlike global proteomics for relative quantification, the two targeted proteomics approaches can provide accurate or absolute protein analysis of small numbers of cells with high reproducibility using heavy isotope-labeled internal standards at known concentrations. When compared to cPRISM-SRM that requires prior high-resolution PRISM fractionation, resulting in many fraction samples that need to be analyzed, cLC-SRM has a significant advantage in sample throughput without fractionation and can simultaneously quantify hundreds of proteins in a single analysis but with relatively lower detection sensitivity[30]. Therefore, cLC-SRM is more accessible and should have broader utilities for accurate multiplexed protein analysis of small numbers of cells as well as mass-limited samples.

Herein we describe a detailed protocol to perform cLC-SRM for convenient targeted proteomics analysis of small numbers of human cells, including single cells. The protocol consists of the following major steps: cell sorting by FACS (fluorescence activated cell sorting), cell lysis and digestion processed in low-volume single polymerase chain reaction (PCR) tubes, LC-SRM data collection, and SRM data analysis using publicly available Skyline software (**Figure 1**). Its broad utility was demonstrated along with our previously well-established SRM assays by absolute targeted quantification of EGFR/MAPK pathway proteins in 1-100 MCF7 or MCF10A cells and determination of pathway protein copies per cell at a wide dynamic range of concentrations[30]. We anticipate that with the detailed protocol most proteomics researchers can readily

implement cLC-SRM in their laboratories for accurate protein analysis of ultrasmall samples (e.g., rare tumor cells) to meet their project needs.

[Place **Figure 1** here]

PROTOCOL:

NOTE: The step-by-step cLC-SRM analysis is shown in **Figure 1**.

1. Pretreatment of PCR tubes

1.1. Add 100 µL of nonhuman (e.g., *Shewanella oneidensis*) cell lysate digests at 0.2 µg/µL to PCR tubes. Incubate at room temperature for overnight to coat PCR tube surface.

1.2. Remove the cell lysate digests by pipetting, rinse PCR tubes with HPLC-grade water 3 times, and then airdry PCR tubes in a fume hood.

1.3. Store coated PCR tubes in a 4 ˚C freezer till further use.

2. FACS sorting

2.1. Align a fluorescence-activated cell sorter (FACS) (see Table of Materials) into a 96-well PCR plate using fluorescent beads with dyes that can be excited by the laser source to provide reliable reference signals.

2.2. Verify that the FACS machine is well aimed for sorting cells into the bottom of PCR plates.

2.2.1. Set the angle of the sort stream to the minimum possible to maximize the success rate that the cells will be sorted into the bottoms of wells rather than hit the sides.

2.2.2. To aim the machine, use the test stream to 'sort' droplets of PBS buffer (e.g., 100 droplets) onto the well of an empty plate to make sure that the collected droplets are on the bottom. If the droplets do not land in the right position, recalibrate the cell sorter and repeat till the droplets in the sort stream are collected correctly.

2.3. Place the coated PCR tubes into a 96-well PCR tube rack.

2.4. Sort desired numbers of human breast cells (MCF7 or MCF10A) into precoated PCR tubes.

2.5. Immediately centrifuge the sorted breast cells at 100 *x g* for 10 min at 4 °C to keep them at the bottom of the tube to avoid potential cell loss.

2.6. Store the sorted cells in a -80 °C freezer until further analysis.

3. Addition of protein carrier, heavy internal standards, and TFE

3.1. Add 4 µL of 25 mM NH_4HCO_3 into PCR tubes containing collected cells.

3.2. Add 1 µL of 10 ng/µL bovine serum albumin (BSA) in 25 mM NH_4HCO_3 into sample tubes.

3.3. Add 0.3 µL of 100 fmol/µL crude heavy peptide standards (total 30 fmol) into sample tubes.

NOTE: Aliquots of crude heavy peptide standards for EGFR/MAPK pathway proteins are made at a nominal concentration of 100 fmol/µL in 25 mM NH_4HCO_3. Dispense the small volume of crude heavy peptide standards at 100 fmol/µL into the middle of the solution and make sure there is no left-over liquid in the pipet tip.

3.4. Add 9 µL of 100% TFE into sample tubes with the final concentration of ~60% TFE.

3.5. Centrifuge at 1500 x g for 5 min and then gently vortex at 100 x g for 3 min.

4. Cell lysis and protein denaturation

4.1. Sonicate FACS-sorted breast cells on ice for 1 min with 1-min intervals for a total of 5 cycles at the 70% amplitude and 0.5 Hz.

4.2. Incubate samples at 90 °C for 1 h for protein denaturation using a PCR thermocycler with the heated-lid option.

NOTE: TFE is a volatile solvent with a boiling point of 74 °C, and thus the heated-lid option is selected to avoid complete drying with substantial sample loss.

4.3. Cool samples to room temperature with centrifugation at 1500 *x g* for 3 min.

5. Reduction and alkylation (optional)

5.1. Add 0.6 µL of 50 mM DTT for a final concentration of 2 mM.

NOTE: DTT solution needs to be freshly prepared each time. Weigh 77.5 mg of DTT and then dissolve into 1 mL of 25 mM NH_4HCO_3 to make 500 mM DTT. 10 µL of 500 mM DTT is then added into 90 µL of 25 mM NH_4HCO_3 to make 50 mM DTT.

5.2. Centrifuge at 1500 *x g* for 5 min and then gently mix at 850 rpm for 3 min.

5.3. Incubate at 56℃ for 1 h with gentle shaking at 100 *x g*.

5.4. Cool samples to room temperature and centrifuge at 1500 *x g* for 3 min.

5.5. Add 0.5 µL of 60 mM IAA to the RT-PCR tube with the final concentration of ~2 mM.

NOTE: IAA solution needs to be freshly prepared each time. Weigh 74.3 mg of IAA and then dissolve into 1 mL of 25 mM NH_4HCO_3 to make 400 mM IAA. 15 µL of 400 mM IAA is added into 85 µL of 25 mM NH_4HCO_3 to make 60 mM IAA.

5.6. Incubate in the dark at room temperature for 30 min with gentle shaking at 100 *x g*.

NOTE: The reduction and alkylation steps are optional when there are no cysteine-containing peptides selected for target proteins.

6. Trypsin digestion

6.1. Reduce the sample volume to ~4 µL using a Speed Vac concentrator.

NOTE: TFE is a volatile solvent with boiling point at 74°C. High percentage TFE inhibits trypsin digestion.

6.2. Add 9 µL of 25 mM NH_4HCO_3 and 1-3 µL of 15 ng/µL trypsin for digestion with the final trypsin concentration of 1-3 ng/µL.

NOTE: For 10 FACS-sorted human breast cells (~1 ng), the ratio of trypsin enzyme over protein is ≥15:1, 750-fold higher than that in standard trypsin digestion (the ratio of 1:50).

6.3. Mix gently at 100 *x g* for 3 min and incubate overnight (~16 hours) at 37 °C.

6.4. Add 0.5 µL of 5% formic acid to stop the enzymatic reaction.

6.5. Centrifuge for 1 h at 1500 *x g*.

6.6. Store in -80 °C freezer until further LC-SRM analysis or in 4 °C freezer for immediate analysis.

7. Preparation for direct LC-SRM analysis

7.1. Remove the cap of the PCR tube that was used for collection (Step 2) and processing (Step 6) of FACS-sorted breast cells.

7.2. Insert PCR tube into LC vial.

7.3. Close the LC vial for LC-SRM analysis.

NOTE: For small numbers of cells processed in 96-well PCR plate, the mat cover will be added to seal the plate for direct LC-SRM analysis.

8. LC-SRM analysis

8.1. Analyze samples using UPLC coupled to a triple quadrupole mass spectrometer.

8.2. Connect capillary C18 columns (75 µm i.d. × 20 cm for standard gradient and 100 µm i.d. × 10 cm for short gradient) to a chemically etched 20 µm i.d. fused silica electrospray emitter via a stainless metal union.

8.3. Use a 20 µL sample loop for directly loading all of the sample (~15 µL in total) into the LC column to maximize detection sensitivity.

8.4. Use 0.1% formic acid in water and 0.1% formic acid in 90% acetonitrile as mobile phases A and B for capillary RPLC separation.

8.5. Use the binary LC gradient at flow rates of 300 nL/min for a standard gradient when large numbers of target proteins (>10) are measured. Standard gradient: 5-20% B in 26 min, 20-25% B in 10 min, 25-40% B in 8 min, 40-95% B in 1 min and at 95% B for 7 min for a total of 52 min and the analytical column re-equilibrated at 99.5% A for 8 min.

8.6. Use the binary LC gradient at flow rates of 400 nL/min for a short gradient when small numbers of target proteins (≤10) are measured. Short gradient for fast separation: 5-95% B in 5 min and the analytical column re-equilibrated at 99.5% A for 5 min with a total 10 min LC run time.

8.7. Operate the QQQ mass spectrometer with ion spray voltages of 2,400 ± 100 V, a capillary offset voltage of 35 V, a skimmer offset voltage of -5 V and a capillary inlet temperature of 220° C.

8.9. Obtain the other QQQ MS parameters from automatic tuning and calibration without further optimization.

8.10. Spike different concentrations of crude heavy peptide standards into 20-50 ng/µL of nonhuman cell lysate digests before LC-SRM analysis of samples to determine peptide LC retention time (RT) for building a scheduled SRM method and potential interference transitions for accurate quantification.

8.11. Use the RT scheduled SRM mode for multiplexed quantification with the scan window of ≥6 min.

NOTE: The scan window will be adjusted accordingly depending on the number of transitions in a certain time window.

8.12. Set the total cycle time to 1 s, and the dwell time for each transition is automatically adjusted depending on the number of transitions scanned at different retention time windows. A minimal dwell time of 10 ms is needed for each SRM transition. All target proteins can be simultaneously monitored in a single LC-SRM analysis

9. Data Analysis

9.1. Import raw data files into publicly available Skyline software[33] for data visualization of each target peptide to determine their detectability.

9.2. Use two criteria to determine SRM peak detection and integration: 1) same LC retention time; 2) approximately the same relative SRM peak intensity ratios across multiple transitions between endogenous (light) peptides and their corresponding (heavy) isotope-labeled peptide internal standards.

9.3. Inspect all the SRM data manually to ensure correct SRM peak assignment and SRM peak boundary for reliable quantification using the above two criteria.

9.4. Use the best transition with a high SRM signal but without matrix interference for protein quantification, and use the other transitions as a reference.

9.5. Use multiple replicates to obtain the standard deviation (SD) and coefficient of variation (CV).

9.6. Calculate the signal-to-noise ratio (S/N) by the peak apex intensity over the average background noise within a retention time region of ±15 s for standard gradient and ±6 s for short gradient. The limit of detection (LOD) and the limit of quantification (LOQ) are defined as the lowest concentration points at which the S/N ratio is at least 3 and 7, respectively.

9.7. Apply one additional criteria for conservatively determining the LOQ values besides S/N ≥7: surrogate peptide response against different cell numbers must be within the linear dynamic range.

9.8. Use the Excel software to plot all calibration curves. Load the RAW data from QQQ MS into publicly available Skyline software to display graphs of extracted ion chromatograms (XICs) of multiple transitions of target proteins monitored.

REPRESENTATIVE RESULTS:

Small amounts of MCF7 cell lysates (0.5-20 ng equivalent to 5-200 cells) were first used to evaluate the performance of cLC-SRM by targeted quantification of EGFR/MAPK pathway proteins because they are more uniform with less variations when compared to small numbers

of cells sorted by FACS. As shown in **Figure 2A**, XICs clearly shows detection of SRM transitions for ATADDELSFK derived from GRB2 present at ~220,000 copies per MCF7 cell[34]. cLC-SRM enabled reproducible quantification of endogenous ATADDELSFK down to 5 MCF7 cell equivalents with a S/N ratio of 14 and ~1,800 zmol of quantification sensitivity. The resultant calibration curves displayed excellent linearity with LOQs of 5 cells for high-abundance GRB2 protein and 20 cells for moderate-abundance PTPN11 protein (**Figure 2B**). The median SRM technical CV for all target peptides across all the data points was ~9%, consistent with the technical reproducibility of standard LC-SRM with CV below 10%[20,23,35-38].

[Place **Figure 2** here]

cLC-SRM was next applied to measure EGFR pathway proteins in 10 and 100 intact MCF7 cells sorted by FACS. cLC-SRM enabled detection of high- and moderate-abundance proteins in 10 intact MCF7 cells (**Figure 2C**). Interestingly, low-abundance AKT1 protein (~3700 copies per MCF7 cell) was also detected with the average S/N ratio of 7, suggesting ~60 zmol of absolute sensitivity of cLC-SRM. With the cell number increased to 100, most previously identified important EGFR pathway proteins (22 out of the total 32 proteins which have a wide dynamic concentration range)[34] were reliably detected and quantified by cLC-SRM. Furthermore, we have recently tested whether a short gradient time (e.g., 5 min *vs* standard 45 min) is sufficient for rapid sensitive cLC-SRM analysis. As shown in **Figure 3**, XICs clearly shows endogenous detection of VLTPTQVK peptide derived from PEBP1 at ~744,000 copies per cell[34] in single MCF10A cells sorted by FACS with a S/N ratio of 5 and ~1,240 zmol of quantification sensitivity. As expected, with the cell number increased to 50 and 75, stronger SRM signal was observed with detection of all three transitions, which have the same pattern as its corresponding heavy internal standard (**Figure 3A**). The calibration curve has linearity with R^2 of 0.89 (**Figure 3B**). Thus, a short gradient is feasible for cLC-SRM presumably because sample complexity for ≤20 ng of tryptic peptides (10 ng BSA and ≤100 human cells ≈ 10 ng proteins) from carrier-assisted small numbers of cells can be effectively addressed by high-resolution capillary RPLC separation with high loading capacity of ≥200 ng. Taken together, these results have shown that cLC-SRM can be used for multiplexed, sensitive, absolute quantification of target proteins in small numbers of human cells including single cells.

[Place **Figure 3** here]

FIGURE LEGENDS:

Figure 1: Overview of all steps in cLC-SRM (carried-assisted one-pot sample preparation coupled with liquid chromatography – selected reaction monitoring). Nonhuman cell lysate digests (e.g., *Shewanella oneidensis*) are used to pretreat PCR tubes for coating tube surface. Small numbers of human cells or single cells sorted by FACS are collected into pretreated PCR tubes. BSA protein (or nonhuman cell lysate proteome) carrier, heavy internal standard (IS), and TFE (or DDM) are added into sample tubes sequentially for facilitating cell lysis and reducing surface adsorption losses. Conceptually, the combined DDM and nonhuman cell lysate proteome carrier will work well for cLC-SRM. Cell lysis is conducted by sonication, and protein denaturation is achieved by heating at high temperature. DTT and IAA reagents are used for reduction and

alkylation, respectively (this step is optional). Trypsin is added for digestion with much higher ratios of trypsin over protein amount than that for standard trypsin digestion. The cap of sample tube is removed and then PCR tube is inserted into LC vial for direct LC-SRM analysis. Collected SRM data are analyzed by using publicly available Skyline software.

Figure 2: Sensitivity and accuracy of cLC-SRM for multiplexed quantification of EGFR/MAPK pathway proteins. (**A**) Extracted ion chromatograms (XICs) of transitions monitored for ATADDELSFK derived from GRB2 at different numbers of MCF7 cell equivalents: 548.8/924.4 (*blue*), 548.8/853.4 (*purple*), 548.8/738.4 (*chestnut*). (**B**) Calibration curves for quantifying high abundance GRB2 and moderate-abundance PTPN11 with the use of the best responsive interference-free transitions, ATADDELSFK (548.8/924.4) for GRB2 and SNPGDFTLSVR (596.9/496.3) for PTPN11. Three and two SRM replicates were performed for 0-10 and 20-200 MCF7 cell equivalents, respectively. (**C**) Comparison of SRM signal between 10 and 100 MCF7 cells sorted by FACS. Each sample consists of two biological replicates with the addition of ~30 fmol of heavy peptide standards per replicate. XICs of transitions monitored for ATADDELSFK derived from GRB2: 548.8/924.4 (*blue*), 548.8/853.4 (*purple*), 548.8/738.4 (*chestnut*); XICs of transitions monitored for FYGAEIVSALDYLHSEK derived from AKT1: 648.0/897.9 (*blue*), 648.0/816.4 (*purple*), 648.0/283.1 (*chestnut*); XICs of transitions monitored for LPSADVYR derived from SOS1: 460.7/807.4 (*blue*), 460.7/710.3 (*purple*), 460.7/404.2 (*chestnut*). This figure has been modified from Zhang et al[30] with the explicit permission from ACS publisher.

Figure 3: An example of targeted proteomics analysis of small numbers of MCF10A cells including single cells with cLC-SRM at short LC gradient. **(A)** Comparison of SRM signal among 1, 50 and 75 MCF10A cells sorted by FACS. ~30 fmol of internal standard was added to each sample. XICs of transitions monitored for VLTPTQVK derived from PEBP1: 443.3/673.4 (*blue*), 443.3/572.3 (*purple*), 443.3/213.2 (*chestnut*). For single cells, the transition 443.3/213.2 was removed due to severe matrix interference. (**B**) Calibration curve for quantifying PEBP1 with the use of the best responsive interference-free transition, VLTPTQVK (443.3/673.4).

DISCUSSION:

cLC-SRM is a convenient targeted proteomics method that enables accurate multiplexed protein analysis of small numbers of cells including single cells. This method capitalizes on protein carrier-assisted one-pot sample preparation, in which all steps including cell collection, multistep cell lysis and digestion, and transfer of peptide digests to capillary LC column for MS analysis are performed in one pot (e.g., single tube or single well) (**Figure 1**). This 'all-in-one' low-volume one-pot processing effectively maximizes the recovery of small numbers of cells for targeted proteomics analysis by greatly reducing possible surface absorption losses.

There are two critical steps for cLC-SRM analysis: 1) after FACS sorting, immediate centrifugation is required to ensure that the collected cells are at the bottom of tubes or wells because a low volume (~15 µL) is used to process small numbers of cells; 2) prior to the addition of trypsin for digestion, the TFE levels need to be reduced down to ≤10% for effective digestion. To avoid drying out samples resulting in unrecoverable loss, frequently checking the sample volume (~4 µL of the remaining volume) is suggested during Speed Vac concentrating. One-pot sample preparation

can be further simplified by removal of reduction and alkylation steps with negligible effect on digestion efficiency. One drawback is that cysteine containing peptides cannot be selected as surrogate peptides for target proteins. To avoid the Speed Vac concentrating step for TFE removal we considered replacing TFE with MS-compatible surfactants (e.g., DDM: n-Dodecyl β-D-maltoside) for cell lysis because frequently checking the sample volume severely prevents automation of sample processing with low throughput. This was evidenced by our recent data for DDM-assisted global single-cell proteomics analysis (unpublished). In addition, a single carrier protein (e.g., BSA) may not be sufficient to reduce surface adsorption losses for every protein without bias. This was evidenced by a few undetected proteins at >30,000 copies per cell (e.g., MAP2K1) in 10 MCF7 cells that should have been detected[30]. Non-human cell lysates presumably can be used as a more effective proteome carrier in contrast to our current BSA carrier because there are many different types of nonhuman proteins (e.g., >3000 proteins for *Shewanella oneidensis*) with less interference for SRM detection of human proteins. We are working on several different ways to further improve cLC-SRM performance with the potential of moving towards sensitive targeted single-cell proteomics.

When compared to other MS-based single-cell proteomics methods that require specific devices, cLC-SRM can be readily implemented in any MS proteomics laboratory without additional investment on specific devices for sample processing. But it can only be used for quantitative analysis of target proteins of interest. Unlike global single-cell proteomics methods for relative untargeted quantification, cLC-SRM can provide accurate or absolute quantification of target proteins in small numbers of cells including single cells. Furthermore, cLC-SRM has significant advantages over conventional targeted single-cell proteomics using antibody-based immunoassays in terms of multiplexing and quantitation specificity and accuracy. Future developments will focus on significant improvements in detection sensitivity and sample throughput for enabling rapid absolute quantification of most proteins (e.g., important negative feedback regulators) in single human cells. Automation of cLC-SRM is another direction to improve its robustness, reproducibility, and the overall sample throughput with commercially available liquid handlers. We anticipate that cLC-SRM will be broadly used for multiplexed accurate quantification of target proteins in small subpopulations of cells and rare tumor cells as well as mass-limited samples. In turn, it will greatly benefit current biomedical research and systems biology.

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DISCLOSURES:

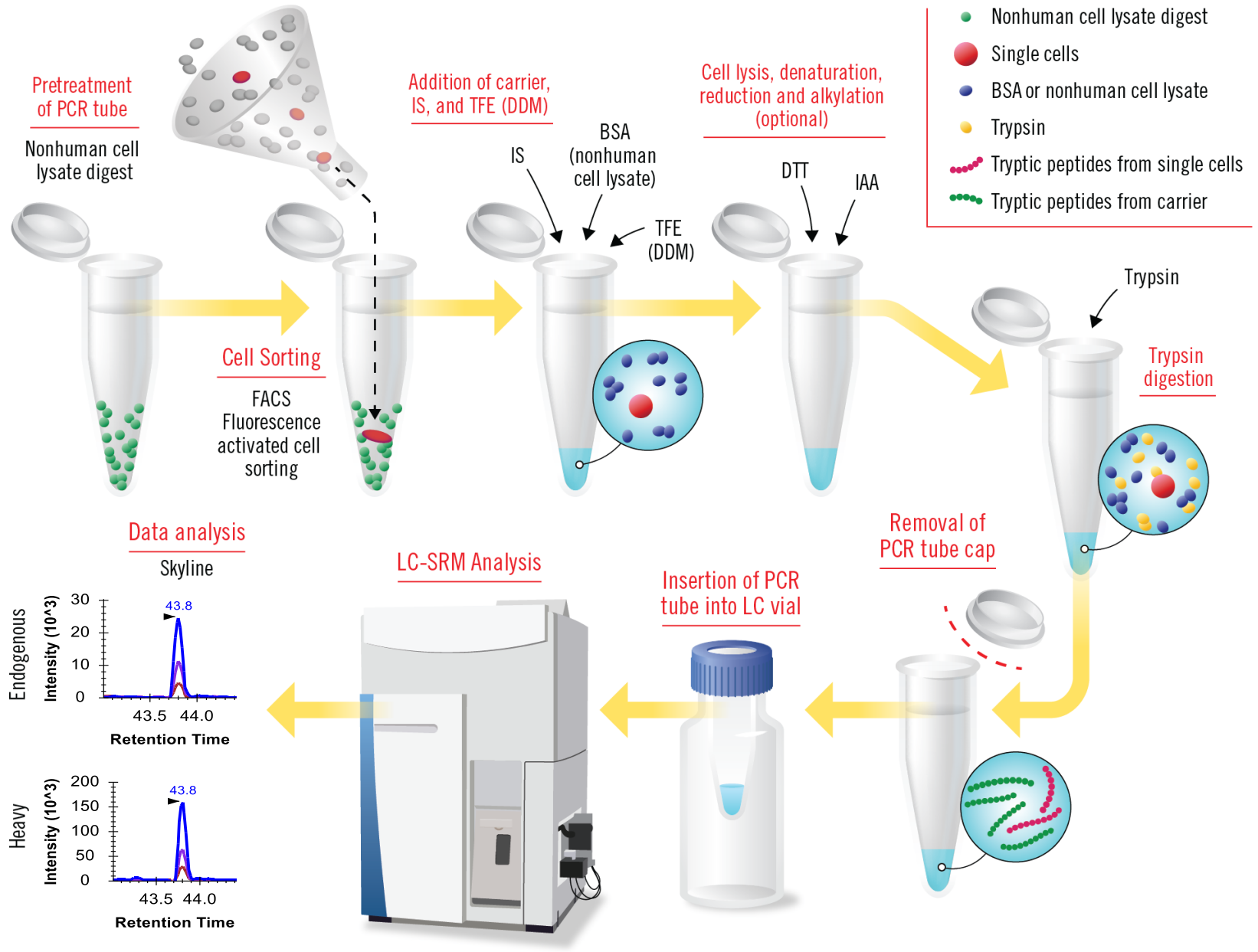
The authors have nothing to disclose.

REFERENCES:

1 Wang, Z., Gerstein, M., Snyder, M. RNA-Seq: a revolutionary tool for transcriptomics. *Nature Reviews Genetics.* **10** (1), 57-63, (2009).
2 Miyamoto, D. T. *et al.* RNA-Seq of single prostate CTCs implicates noncanonical Wnt signaling in antiandrogen resistance. *Science.* **349** (6254), 1351-1356, (2015).
3 Wang, Y., Navin, N. E. Advances and applications of single-cell sequencing technologies. *Molecular Cell.* **58** (4), 598-609, (2015).
4 Macaulay, I. C., Ponting, C. P., Voet, T. Single-Cell Multiomics: Multiple Measurements from Single Cells. *Trends in Genetics.* **33** (2), 155-168, (2017).
5 Gavasso, S., Gullaksen, S. E., Skavland, J., Gjertsen, B. T. Single-cell proteomics: potential implications for cancer diagnostics. *Expert Review of Molecular Diagnostics.* **16** (5), 579-589, (2016).
6 Wu, M., Singh, A. K. Single-cell protein analysis. *Current Opinion in Biotechnology.* **23** (1), 83-88, (2012).
7 Hughes, A. J. *et al.* Single-cell western blotting. *Nature Methods.* **11** (7), 749-755, (2014).
8 Peterson, V. M. *et al.* Multiplexed quantification of proteins and transcripts in single cells. *Nature Biotechnology.* **35** (10), 936-939, (2017).
9 Liu, Y., Beyer, A., Aebersold, R. On the Dependency of Cellular Protein Levels on mRNA Abundance. *Cell.* **165** (3), 535-550, (2016).
10 Choudhary, C., Mann, M. Decoding signalling networks by mass spectrometry-based proteomics. *Nature Reviews Molecular Cell Biology.* **11** (6), 427-439, (2010).
11 Mertins, P. *et al.* Proteogenomics connects somatic mutations to signalling in breast cancer. *Nature.* **534** (7605), 55-62, (2016).
12 Zhang, H. *et al.* Integrated Proteogenomic Characterization of Human High-Grade Serous Ovarian Cancer. *Cell.* **166** (3), 755-765, (2016).
13 Zhang, B. *et al.* Proteogenomic characterization of human colon and rectal cancer. *Nature.* **513** (7518), 382-387, (2014).
14 Gaudet, S., Miller-Jensen, K. Redefining Signaling Pathways with an Expanding Single-Cell Toolbox. *Trends in Biotechnology.* **34** (6), 458-469, (2016).
15 Giesen, C. *et al.* Highly multiplexed imaging of tumor tissues with subcellular resolution by mass cytometry. *Nature Methods.* **11** (4), 417-422, (2014).
16 Di Palma, S., Bodenmiller, B. Unraveling cell populations in tumors by single-cell mass cytometry. *Current Opinion in Biotechnology.* **31**, 122-129, (2015).
17 Schulz, K. R., Danna, E. A., Krutzik, P. O., Nolan, G. P. Single-cell phospho-protein analysis by flow cytometry. *Current Protocols in Immunology.* **Chapter 8** Unit 8 17 11-20, (2012).
18 Willison, K. R., Klug, D. R. Quantitative single cell and single molecule proteomics for clinical studies. *Current Opinion in Biotechnology.* **24** (4), 745-751, (2013).
19 Lee, J. Y. *et al.* Detection of Head and Neck Cancer Based on Longitudinal Changes in Serum Protein Abundance. *Cancer Epidemiology, Biomarkers & Prevention.* **29** (8), 1665-1672, (2020).
20 Shi, T. *et al.* Advancing the sensitivity of selected reaction monitoring-based targeted quantitative proteomics. *Proteomics.* **12** (8), 1074-1092, (2012).
21 Shi, T. *et al.* Advances in targeted proteomics and applications to biomedical research. *Proteomics.* **16** (15-16), 2160-2182, (2016).

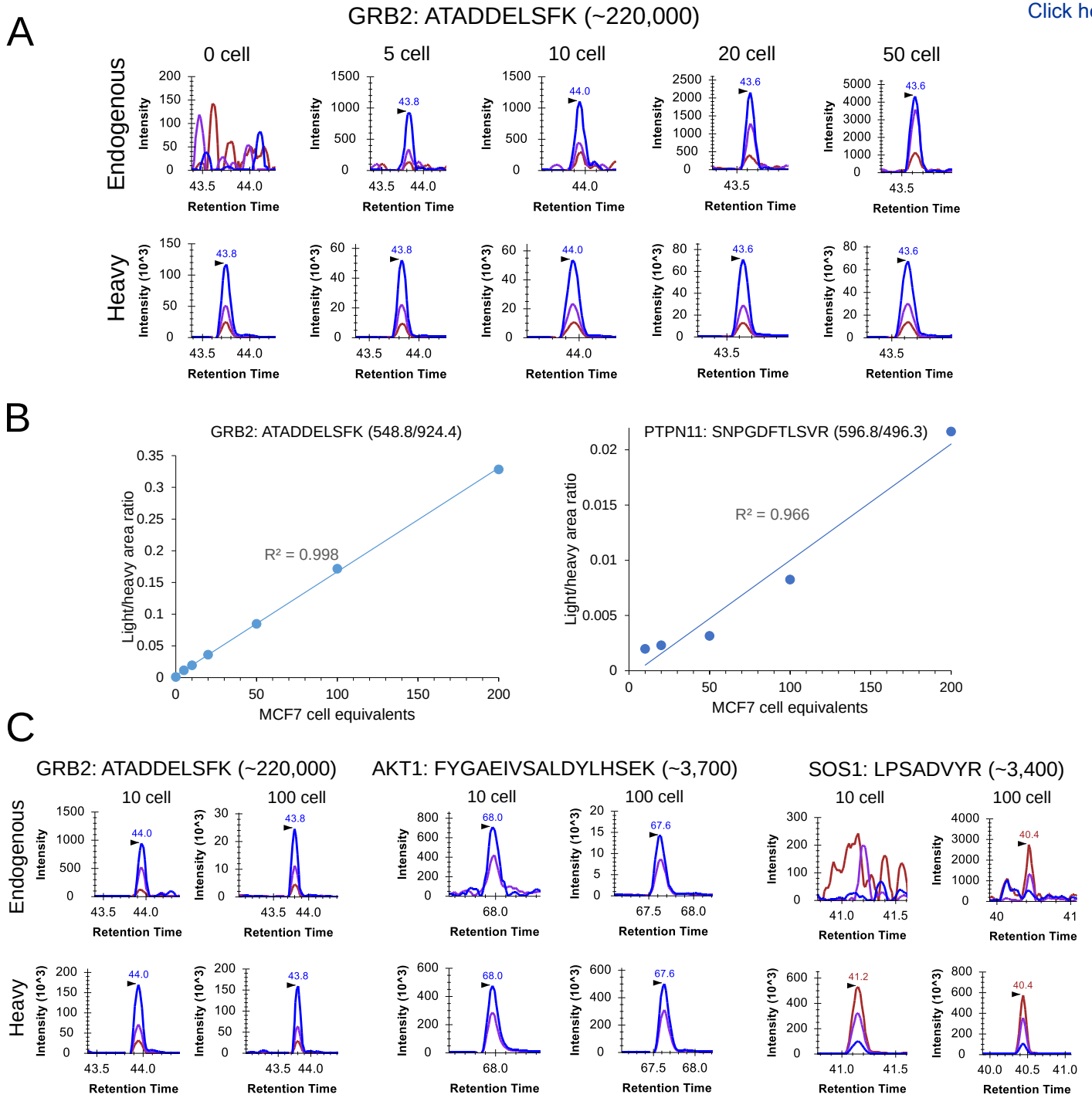
22 Lange, V., Picotti, P., Domon, B., Aebersold, R. Selected reaction monitoring for quantitative proteomics: a tutorial. *Molecular Systems Biology.* **4**, 222, (2008).
23 Picotti, P., Aebersold, R. Selected reaction monitoring-based proteomics: workflows, potential, pitfalls and future directions. *Nature Methods.* **9** (6), 555-566, (2012).
24 Zhu, Y. *et al.* Proteomic Analysis of Single Mammalian Cells Enabled by Microfluidic Nanodroplet Sample Preparation and Ultrasensitive NanoLC-MS. *Angewandte Chemie International Edition.* **57** (38), 12370-12374, (2018).
25 Cong, Y. *et al.* Improved Single-Cell Proteome Coverage Using Narrow-Bore Packed NanoLC Columns and Ultrasensitive Mass Spectrometry. *Analytical Chemistry.* **92** (3), 2665-2671, (2020).
26 Zhu, Y. *et al.* Nanodroplet processing platform for deep and quantitative proteome profiling of 10-100 mammalian cells. *Nature Communications.* **9** (1), 882, (2018).
27 Shao, X. *et al.* Integrated Proteome Analysis Device for Fast Single-Cell Protein Profiling. *Analytical Chemistry.* **90** (23), 14003-14010, (2018).
28 Li, Z. Y. *et al.* Nanoliter-Scale Oil-Air-Droplet Chip-Based Single Cell Proteomic Analysis. *Analytical Chemistry.* **90** (8), 5430-5438, (2018).
29 Budnik, B., Levy, E., Harmange, G., Slavov, N. SCoPE-MS: mass spectrometry of single mammalian cells quantifies proteome heterogeneity during cell differentiation. *Genome Biology.* **19** (1), 161, (2018).
30 Zhang, P. *et al.* Carrier-Assisted Single-Tube Processing Approach for Targeted Proteomics Analysis of Low Numbers of Mammalian Cells. *Analytical Chemistry.* **91** (2), 1441-1451, (2019).
31 Shi, T. *et al.* Facile carrier-assisted targeted mass spectrometric approach for proteomic analysis of low numbers of mammalian cells. *Communications Biology.* **1**, 103, (2018).
32 Vitrinel, B., Iannitelli, D. E., Mazzoni, E. O., Christiaen, L., Vogel, C. Simple Method to Quantify Protein Abundances from 1000 Cells. *ACS Omega.* **5** (25), 15537-15546, (2020).
33 MacLean, B. *et al.* Skyline: an open source document editor for creating and analyzing targeted proteomics experiments. *Bioinformatics.* **26** (7), 966-968, (2010).
34 Shi, T. *et al.* Conservation of protein abundance patterns reveals the regulatory architecture of the EGFR-MAPK pathway. *Science Signaling.* **9** (436), rs6, (2016).
35 Addona, T. A. *et al.* Multi-site assessment of the precision and reproducibility of multiple reaction monitoring-based measurements of proteins in plasma. *Nature Biotechnology.* **27** (7), 633-641, (2009).
36 He, J. *et al.* Analytical platform evaluation for quantification of ERG in prostate cancer using protein and mRNA detection methods. *Journal of Translational Medicine.* **13**, 54, (2015).
37 Shi, T. *et al.* Antibody-free, targeted mass-spectrometric approach for quantification of proteins at low picogram per milliliter levels in human plasma/serum. *Proceedings of the National Academy of Sciences.* **109** (38), 15395-15400, (2012).
38 Shi, T. *et al.* Targeted quantification of low ng/mL level proteins in human serum without immunoaffinity depletion. *Journal of Proteome Research.* **12** (7), 3353-3361, (2013).

Figure

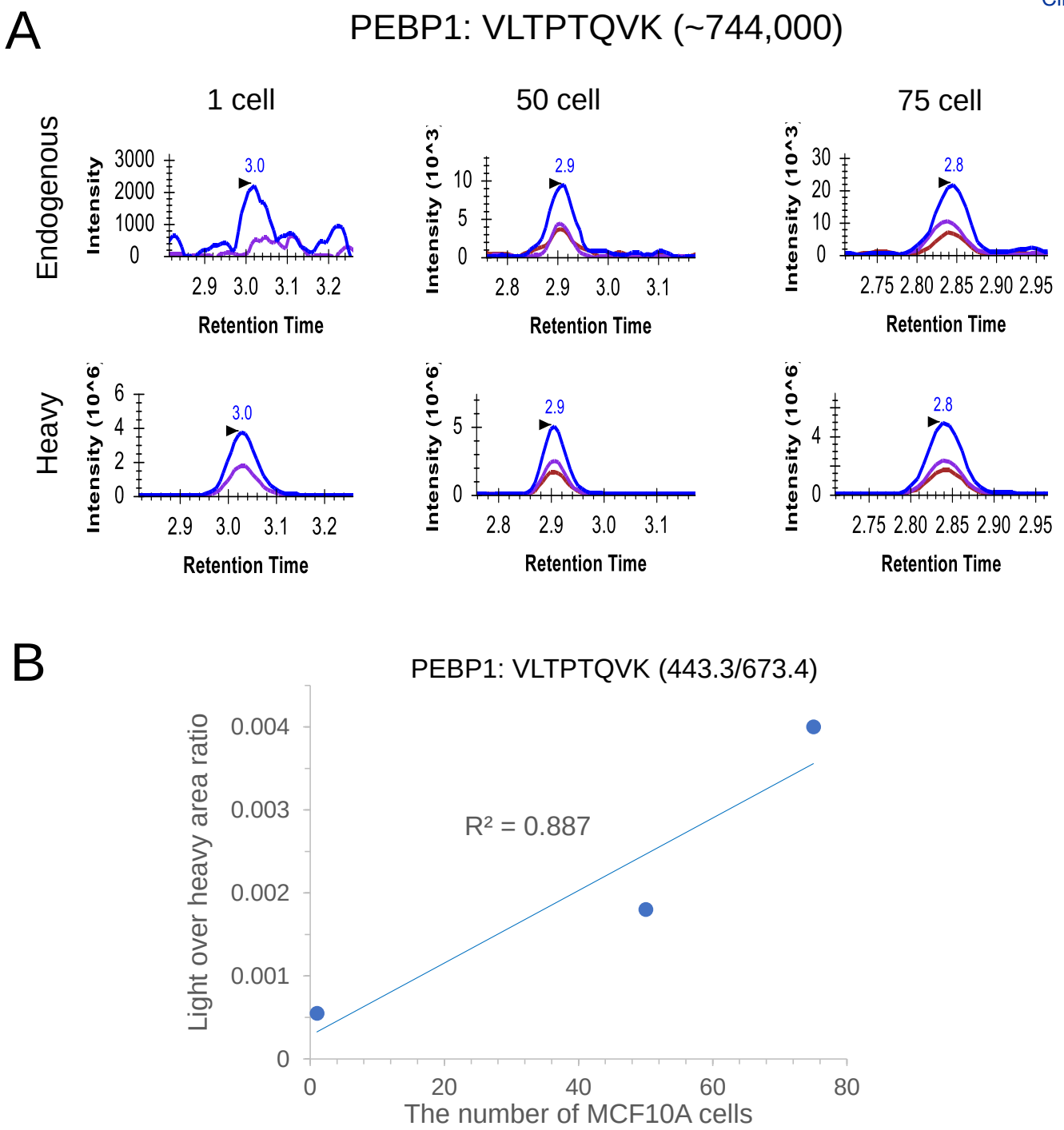


Figure

Click here to access/download;Figure;Figure 2.pdf



Figure



Name of Material/Equipment	Company	Catalog Number
2 mL glass LC vial	Microsolv	9502S-WCV
BSA	Sigma-Aldrich	P0834-10×1mL
DTT	Thermo Scientific	A39255
Formic acid	Thermo Scientific	28905
IAA	Thermo Scientific	A39271
Peptide internal standards	New England peptide	
RT-PCR tube	GeneMate Bioexpress	T-3035-1
Skyline software	University of Washington	
Sonicator	Technology	UTR200
Speed Vac concentrator	Thermo Scientific	
TFE	Sigma-Aldrich	18370-10×1mL
Thermocycler w/ heated lid	Peltier Thermal Cycler	PTC-200
Trypsin Gold	Promega	V528A
Waters BEH C18 column	Waters	

Comments/Description
Vessel to hold PCR tube for autosample injection
Carrier protein for greatly reducing surface adsorption losses
Reagent for reduction
Reagent for stopping enzyme reaction
Reagent for alkylation
Targeted quantification of EGFR/MAPK pathway proteins
0.2 mL PCR tube for one-pot sample preparation
Publicly available for SRM data analysis
Sonication on ice for cell lysis
Reduction of the percentage of TFE for effective trypsin digestion
60% TFE for cell lysis
Heating for protein denaturation
Enzyme for protein digestion
C18 column for peptide separation

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October 14, 2020

Vineeta Bajaj, Ph.D.
Review Editor
JoVE
Tel: (617)674-1888
Email: vineeta.bajaj@jove.com

Dear Dr. Bajaj,

Enclosed is our revised manuscript (JoVE61797) entitled “Carrier-assisted One-pot Sample Preparation for Targeted Proteomics Analysis of Small Numbers of Human Cells”.

We appreciate the prompt review of our manuscript and the constructive comments from you and the reviewers. We have revised the manuscript carefully to address all the concerns and suggestions. We apologize that it took us so long to finish this resubmission.

We believe that the revisions made to the manuscript have appropriately addressed all of the comments, and improved the overall clarity and readiness of the paper. I hope that you will find the revised manuscript suitable for publication

Sincerely yours,

Shi Tujin

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Point-by-Point Response to the Reviewers' Comments

(The Reviewer's original comments were in *italics*, and response in dark blue)

Editorial Comments:

• *Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.*

Response: We thank the Editor for this comment. We have thoroughly checked the manuscript to ensure no spelling or grammar errors.

• *Please list a minimum of 6 keywords/phrases.*

Response: Key words have been added.

• *Textual Overlap: Significant portions show significant overlap with previously published work. Please re-write lines 60-65, 104-107, 134-136, 191-197, 205-207, 244-248, 259-261, 270-272, 275-279, 288-291, 297-299, 301-305, 306-314, 352-372, 406-408, avoid this overlap.*

Response: We thank the Editor for arising this concern and relevant changes have been made to avoid significant overlap with previously published work.

• *Protocol Language: Please ensure that ALL text in the protocol section is written in the imperative voice/tense as if you are telling someone how to do the technique (i.e. "Do this", "Measure that" etc.) Any text that cannot be written in the imperative tense may be added as a "Note", however, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Some examples NOT in the imperative:*

1) 8.2, 8.5, 8.6, 8.7, 8.8, 8.9, 8.10, 9.6-9.8

Response: We have looked through the protocol section and relevant changes have been made to ensure that imperative voice/tense was used in the protocol.

• *Protocol Detail: 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 add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Some examples:*

1) 1.2: How are they removed?

2) 2.1: How is the alignment performed?

3) 2.5, 3.5,5.2, 6.5, etc: Convert the speed to g.

4) 4.1: Mention sonication amplitude (in Watt) and frequency (in Hz).

Response: Relevant changes have been made in the revised manuscript.

• *Protocol Numbering:*

1) Please adjust the numbering of your protocol section to follow JoVE's instructions for authors, 1. should be followed by 1.1. and then 1.1.1. if necessary.

2) All steps should be lined up at the left margin with no indentations.

3) Please add a one-line space after each protocol step.

Response: We have formatted the manuscript accordingly.

• *Protocol Highlight: Please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.*

Response: We have highlighted the protocol with focusing on sample processing steps.

1) The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.

Response: All relevant details are included for the highlighted steps.

2) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.

Response: The order of these highlighted steps essentially contains the logical flow (pretreatment of PCR tubes – FACS cell sorting – the addition of carrier, internal standards and TFE – cell lysis and protein denaturation – reduction and alkylation – trypsin digestion – preparation for direct LC-SRM analysis).

3) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.

Response: Relevant highlights have been made in the revised manuscript.

4) Notes cannot be filmed and should be excluded from highlighting.

Response: We thank the Editor for pointing out this.

• *Discussion: JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.*

Response: We have followed this guideline for preparation of the section of Discussion.

• *References: Please spell out journal names.*

Response: Journal full names have been spelled out.

• *Commercial Language:JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are BD Influx flow cytometer, Speed Vac, a nanoACQUITY UPLC® 259 (Waters Corporation, TSQ Vantage triple quadrupole mass spectrometer (Thermo Scientific, etc.*

1) Please use MS Word's find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names.

Response: All commercial sounding language has been removed in the revised manuscript.

• *Table of Materials:*

1) Please sort in alphabetical order.

Response: We have changed the order (*please see the revised table for details*).

• *Please define all abbreviations at first use (e.g, BSA, PCR, etc)*

Response: All the abbreviations have been defined when they appeared for the first time.

• *If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in*

the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

Response: We have reused **Fig. 2** which originated from Analytical Chemistry (ACS publications) with modifications suggested by the Reviewer. We have obtained the explicit permission from ACS publisher with the link (https://s100.copyright.com/AppDispatchServlet) and uploaded the pdf file to the Editor Manger site in the 'Supplemental Files' section. The sentence 'This figure has been modified from Zhang et al[30] with the explicit permission from ACS publisher' has been added in the end of **Fig. 2** legend.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

In the manuscript, the authors present a detailed protocol for cLC-SRM analysis of proteins in small numbers of human cells. The protocol includes cell sorting, cell lysis and protein digestion, LC-SRM analysis, and data analysis. MS-based proteomics has become very powerful to globally analyze proteins. However, it is very challenging for effective processing of single cells with minimal sample loss prior to MS analysis. The authors have recently developed a convenient protein carrier-assisted one-pot sample preparation method coupled with liquid chromatography (LC) - selected reaction monitoring (SRM) for targeted proteomics analysis of small numbers of human cells including single cells. Using the combined excessive exogenous protein as a carrier and low-volume one-pot processing, they greatly reduced the sample loss. High-specificity of LC-SRM can effectively address the issue about the increased dynamic concentration range resulted from the addition of exogenous carrier protein. This method is very useful for proteomics analysis of limited amount of samples. Many scientists will benefit from this useful method and protocol presented in this manuscript.

Major Concerns:

None

Response: We thank the Reviewer for this positive comment.

Minor Concerns:

"NOTE: 10 µL of 500 mM DTT is added into 90 µL of 25 mM NH4HCO3 to make 50 mM DTT." Because DTT is solid, it might be better to use the weight of DTT. It might also be better to add: the solution needs to be freshly prepared. The same is for IAA.

Response: Relevant changes have been made in the section of Reduction and alkylation.

"High percentage TFE inhabits trypsin digestion." Here it should be "inhibits".

Response: We have changed the wrong spelling.

Reviewer #2:

Manuscript Summary:

Martin et al provide a manuscript accompanying a to-be-produced video protocol for cLC-SRM (carried-assisted one-pot sample preparation coupled with liquid chromatography - selected reaction monitoring). This builds upon earlier work by the authors from 2018 (doi: 10.1038/s42003-018-0107-6) that describes a proof of principle for the approach of using an excess of carrier protein (such as BSA or a complex lysate of a different species) to facilitate sample preparation of small numbers of cells for proteomic analysis with minimal sample loss.

Single-cell proteomics approaches are in their infancy, and innovations in sample preparation will be instrumental to advance the field. A video protocol will be of great use for the proteomics community.

The authors demonstrate that they can detect transitions for selected proteins of medium to high abundance and that the intensity ratio over a heavy-labeled spike-in standard correlated with the amount of input material, both from cell lysate equivalent to small numbers of cells, as well as from samples actually prepared from small numbers of cells. Finally, the authors show that very short LC gradients (5 min) can be sufficient to detect signal from small cell numbers for abundant proteins.

General comments:

1) The reviewer feels that manuscript in its current form would benefit from a greater focus on the practical aspects of readers trying to replicate this type of sample preparation (rather than, e.g. on further optimization of the protocol, such as shortening the gradients).

There are lots of optional or alternative steps contained in the protocol and presented with a high degree of ambiguity. To maximize the utility of the protocol, these need to be not just listed but rather presented to the reader in the form of a decision tree.

Specifically:

Depending on the properties of the sample or the desired outcome, when should the reader skip the optional reduction/alkylation step? Is having/not having cysteines in the target peptides the main/only criterion?

Response: We thank the Reviewer for raising these questions. We have added a note in the subsection of Reduction and alkylation: The reduction and alkylation steps are optional when there are no cysteine-containing peptides selected for target proteins. In addition, in the section of Discussion one sentence is used for additional explanation: 'One drawback is that cysteine containing peptides cannot be selected as surrogate peptides for target proteins'.

Similarly, the use of DDM vs TFE is discussed very vaguely, leaving the reader more confused than supported in executing the protocol.

What are the benefits/drawbacks of using a non-human carrier lysate over using BSA? How should the carrier lysate be prepared?

Response: We agree with the Reviewer. It is vague for discussion of DDM versus TFE. In the section of Discussion, we have added one sentence that 'We are working on several different ways to further improve cLC-SRM performance with the potential of moving towards sensitive targeted single-cell proteomics.' DDM is one way to be explored. Another way is to replace single BSA protein with non-human cell lysate as a carrier because single BSA protein may not be sufficient for unbiasedly reducing surface adsorption losses for every protein.

For digestion, samples are to be incubated "at 75-90 °C". This is a huge temperature range and it is unclear what the implications are of picking temperatures towards the extremes of that range.

Response: We thank the Reviewer for point out this issue. We have changed the range of temperature into 90 °C to avoid such confusion.

2) The authors present the potential of the applicability of their protocol to the analysis of single cells as one of the main selling points of their protocol. (The term "single cell" is mentioned 6 times in the abstract.) However, the reviewer was somewhat sobered to find that there is only one piece of data actually from single cells (in Fig. 3). Also, in this particular instance, some data had to be removed "due to severe matrix interference" (what is that?). The reviewer thinks that it is therefore appropriate to tone down the emphasis on single cells. (The title of the manuscript was appropriate in this regard.)

Response: We agree with the Reviewer. In the section of Abstract we have reduced the number of using the term of 'single cell' down to 3 (*please see the revised manuscript*). In **Fig. 3** the lowest abundance transition (*chestnut*) has been removed due to significant SRM signal contributed by matrix (background) interference and the highest abundance SRM transition (*blue*) was used for quantification.

*3) In Fig. 2 the calibration curves are calculated for samples prepared from lysate *corresponding to* defined numbers of cells, not actually from single cells. This is misleading for a number of reasons: First, it is unclear how these samples were prepared as the protocol only discusses the preparation of samples from actual individually sorted cells. Second, using data from amounts of lysate corresponding to certain cell numbers likely yield a much higher correlation of signal to input compared to data from actual single cells, because of the inherently less robust sample preparation, in addition to cell-to-cell fluctuations in protein abundance. It is important for the reader to get a realistic expectation of the power of the method by showing correlations of cell numbers (input) to signal (output). The most straightforward way is to extract the ratios from the 10 and 100 cell samples in panel C and prepare similar plots as presented in panel B. These would only have 2 data points for each transition (i.e. correlation = 1), but one could observe the deviation from a trendline going through the origin.*

Response: We thank the Reviewer for this constructive comment. However, we do not agree with the Reviewer that it is misleading to use cell lysates for initial performance evaluation. As mentioned in the section of Representative results, when compared to small numbers of cells

sorted by FACS, small amounts of cell lysates (e.g., 0.5-20 ng equivalent to 5-200 cells) are more uniform with less variations, which would be perfect for evaluation of the cLC-SRM method. We agree with the Reviewer that cell lysates should give much higher correlation between SRM signal and low-mass input when compared to actual small numbers of cells (e.g., robust processing and cell-to-cell variations). Due to only 2 data points for 10 and 100 cells we then turned to plot the 3 data points from the short gradient though the SRM data is preliminary with one replicate for each cell number (**Fig. 3B**). As expected, we do observe the trendline of SRM signal against the cell number.

4) The preparation of the spike-in reference standard is not discussed and the procedure of calibrating crude heavy peptide concentrations with high-purity light peptides needs to be expanded on (at least referenced).

Also, from a practical point of view, adding a volume of 0.3ul of standard seems prone to rather large pipetting errors which will carry over to the quantitative accuracy of the method.

Making a master mix of NH4HCO3 /BSA/reference standard and adding a larger volume could be an option here.

Response: One note has been added to the protocol about how to prepare the spike-in reference standard and how to add a volume of 0.3 µL of peptide standard. To avoid confusion, we have removed the content for calibrating crude heavy peptide concentrations because pure heavy peptide standard is commercially available but with higher cost than crude peptide standard. We appreciate the insightful suggestion made by the Reviewer for making a master mixture and will consider this in our future work.

Minor:

1) Fig. 1: What is "IS"?

Response: 'IS' is 'Internal Standard'. Relevant change has been made in the revised manuscript.

2) Line 265. unclear statement: "directly loading all the samples".

Should probably read: "directly loading all of the sample" or "directly loading the entire sample"

Response: We appreciate this suggestion made by the Reviewer. We have modified 'directly loading all the samples' into 'directly loading all of the sample'.

3) The transition plots (Figs. 2 and 3) need to be improved.

All axes need to be geometrically aligned with each other.

The axes also need to be equally scaled where appropriate:

Retention time ranges have to be exactly (not just roughly) the same across corresponding plots.

For the heavy reference peptides, the scales need to be equal across cell numbers, rather than scaling all peaks to the same height with varying intensity ranges. This will provide a sense of the reproducibility of the spike-in standard across samples.

The fonts for axis labels are sometimes distorted.

Many labels are redundant, i.e. unnecessarily plotted multiple times.

Lines of the colors need to be defined in a legend that is part of the figure, not just in the text of the figure legend.

Gene names should be listed in the figure and "copy number" appended to make clear what the approximate numbers refer to.

Response: We thank the Reviewer for all these constructive comments. However, the SRM transition plots in **Figs. 2-3** were automatically generated by using the most popular publicly available Skyline software and we cannot change the plotting format of the two figures. Gene name has been included in **Figs. 2-3** (*please see the revised **Figs. 2-3***).

Reviewer #3:

The authors provide a protocol for a method (cLC-SRM) that they published 2 years ago.

The manuscript omits important competing methods and mischaracterizes others. Below are a few examples:

-- The authors did not mention the nanoliter-scale oil-air-droplet (OAD) chip (Anal. Chem. 2018, 90, 5430−5438, DOI: 10.1021/acs.analchem.8b00661) and other methods which in some ways outperform their method.

Response: We thank the comments raised by the Reviewer. The OAD chip method has been mentioned in the section of Introduction in the revised manuscript. There are few methods reported for proteomic analysis of single mammalian cells (Marx *et al Nat Methods* 2019, 16, 809) and most methods require specific devices that cannot be readily accessible by research community. For example, the OAD chip method that the Reviewer mentioned is difficult to be adopted by other researchers without expertise in microfluidics. There is no direct comparison in performance between other and our methods, and thus it is hard to determine the performance of other methods for targeted proteomics analysis of small numbers of cells. Furthermore, performance comparison is beyond the scope of this manuscript.

-- The authors wrote that antibody methods allow for low multiplexing. However, in terms of demonstrated capability, the cLC-SRM method described by the authors has much lower multiplexing capabilities and lower throughput compared to antibody based methods, such as CyTOF.

Response: It is well known that antibody-based methods have low multiplex (≤30 target proteins) and limited specificity. SRM-based targeted proteomics has been demonstrated for

enabling quantification of ≥150 proteins in a single analysis with high specificity (Lee *et al Cancer Epidemiol Biomarkers Prev.* 29(8),1665-1672 (2020); Thermo Scientific. *https://assets.thermofisher.com/TFS-Assets/CMD/Reference-Materials/pp-qqq-ms-altis-quantis-msum2017-en.pdf*). Therefore, cLC-SRM has much higher multiplex than antibody-based methods though in this manuscript it was only used for simultaneous quantification of 32 target proteins. We agree that cLC-SRM has much lower sample throughput when compared to antibody-based methods.

-- The authors mischaracterize the advantages of the isobaric carrier approach (DOI: 10.1016/j.cbpa.2020.04.018) and the accuracy and reproducibility of SCoPE-MS. Both the original publication and the reproduction of the isobaric carrier by some of the authors (DIO: 10.1021/acs.analchem.9b03349 and DIO: 10.1074/mcp.RA119.001857) demonstrate reproducibility at r = 0.99 and accuracy at r = 0.7 - 0.8.

Response: We respectfully disagree with the Reviewer. In the section of Introduction we have clearly stated that SCoPE-MS (*DOI: 10.1016/j.cbpa.2020.04.018*) has low reproducibility with a correlation coefficient of only ~0.2-0.4 (Vitrinel *et al ACS Omega* 2020, 5, 25, 15537) between replicates because it cannot effectively prevent surface adsorption losses when single tube was used for single-cell processing without any protection. However, this does not rule out other isobaric carrier approaches. For example, the nanoPOTS platform has been well demonstrated for effective processing of single cells by downscaling sample processing volume down to ~200 nL for label-free global proteomic analysis and high reproducibility for TMT-based relative quantification (i.e., isobaric carrier) with correlation coefficient of 0.9 (*DIO: 10.1021/acs.analchem.9b03349*, Dou *et al Anal Chem* 2019, 91, 13119; *DIO: 10.1074/mcp.RA119.001857*, Tsai *et al Mol Cell Proteomics* 2020, 19, 828).

Some statements are inaccurate. Below are a few examples:

-- "cLC-SRM was demonstrated to enable multiplexed accurate quantification of most moderately abundant proteins in small numbers of cells (e.g., 10-100 cells)..." cLC-SRM has been demonstrated to quantify only a few peptides, which is hardly "most moderately abundant proteins".

Response: Please check Table 2 for detected pathway proteins from Zhang *et al Anal Chem* 2019, 91, 1441, which has clearly indicated that most moderately abundant proteins can be detected by cLC-SRM. A set of surrogate peptides which correspond to EGFR/MAPK pathway proteins with a range of different protein abundance (i.e., protein copies per cell) were used to evaluate its detection sensitivity in small numbers of cells. Based on SRM analysis we concluded that it has the ability to detect and quantify target proteins at moderate abundance from small numbers of cells.

-- The authors have many statements about "a wide dynamic range of concentrations", which I find misleading because I do not consider these ranges wide. It will be best if they specify with a number what that range, e.g., 10-fold. That fact that they find a 10-fold wide dynamic range is less useful information for the readers.

Response: We have clearly mentioned ‘a wide dynamic range of concentrations’ rather than the cell number. Please check Table 2 for detected pathway proteins (the highest abundance PEBP1: 1,245,120 copies per cell; the lowest abundance GAB1: 2867 copies per cell) from Zhang *et al Anal Chem* 2019, 91, 1441, which has indicated the quantitation dynamic range in protein concentrations.

-- "Recent technological advances in genomics (transcriptomics) allow for precise measurements for precise measurements of whole genome (transcriptome) in single cells 1-3." This overstates the capabilities of sequencing methods and is inaccurate.

Response: Relevant change has been made in the revised manuscript.

Many limitations of cLC-SRM are not clearly stated, for example the FACS sorting requires that the starting material is large enough for FACS sorting.

Response: We respectfully disagree with the Reviewer. cLC-SRM is not only limited to small numbers of cells or single cells from FACS sorting, but it can also be used for analysis of other types of small samples (e.g., isolated circulating tumor cells from patient blood or LCM-dissected small tissue voxels). Furthermore, FACS can also be used to isolate rare populations of tumor cells from large numbers of normal cells for cLC-SRM analysis. In the section of Discussion, we have clearly discussed the limitations of current cLC-SRM and future improvement.

Reviewer #4:

Manuscript Summary:

The manuscript provides a good step by step protocol to perform the proposed one-pot sample preparation - the authors give advice on critical steps and reproducing the experiments should not be a problem for anyone who worked with SRM assays before. The manuscript very well explains benefits and drawbacks of the proposed method in comparison to state of the art approaches. Additionally, the authors show a possible solution to their low sample throughput in contrast to other methods.

In general the protocol and the introduction need to be revised - several missing words, phrasing or spelling mistakes.

Response: We thank the Reviewer for this positive comment. Relevant changes have been made in the revised manuscript.

Minor Concerns:

The authors stress, that their method 'can be easily implemented in any MS and proteomics laboratories at no additional cost for instrument or reagents'. However, the synthesis of heavy peptides might pose a challenge to some labs and indeed requires additional reagents.

Response: We respectfully do not agree with the Reviewer. Heavy isotope-labeled peptide standards are commonly used for MS-based targeted proteomics and they are commercially available from multiple vendors (e.g., Thermo Scientific and New England peptide) with low cost for crude heavy peptides (~$30 per peptide).

In the text the authors often refer to a peptide shown in Fig 2 or 3 according to its derived protein name, the addition of the respective protein name to the plots would increase readability. Additionally, in Fig 2 A I suggest adding the respective peptide/protein labeling even though it is just one peptide.

Response: We thank the Reviewer for this suggestion. Relevant changes have been made in the revised **Figs. 2-3**.

In Figure 2B it would be beneficial to add error bars to the data points, as all the measurements were acquired in technical triplicates and high reproducibility is proposed.

Response: We agree with the Reviewer. However, not every cell number equivalent has three replicates: three and two SRM replicates were performed for 0-10 and 20-200 MCF7 cell equivalents, respectively (Zhang *et al Anal Chem* 2019, 91, 1441). Thus, we have decided not to add error bars to the data points.

The short gradient experiments already improve one of the major drawbacks of the method, to achieve a direct comparison between the 'standard' and the short gradient I recommend a side by side comparison of the peptide shown in Fig 3 for both gradient times. Additionally, if available for the short gradient experiments displayed in Fig 3, a 100 cell analysis would be the ideal comparison to Fig 2C in terms of sensitivity.

Response: We agree with the Reviewer and thank the Reviewer for these suggestions. Unfortunately, the data from short gradients are still preliminary and we do not have the side by side comparison data (e.g., different numbers of cells at both short and standard gradients). We are working on further improving cLC-SRM performance for analysis of small numbers of cells including single cells using short LC gradients (*to be published elsewhere*).

Carrier-Assisted Single-Tube Processing Approach for Targeted Proteomics Analysis of Low Numbers of Mammalian Cells

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