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# Carrier-assisted One-pot Sample Preparation for Targeted Proteomics Analysis of Small Numbers of Human Cells V.2

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**Protocol status:** Working**We use this protocol and it's working****Created:** April 20, 2024**Last Modified:** April 20, 2024**Protocol Integer ID:** 98529**Keywords:** Protein Carrier, Carrier-assisted One-pot Sample Preparation, cLC-SRM, Small Numbers of Cells, Single Cells, Mass-limited Sample, Mass Spectrometry-based Targeted Single-cell Proteomics**Funders Acknowledgement:****NIH****Grant ID:** UC4 DK104167

## Abstract

Protein analysis of small numbers of human cells is primarily achieved by targeted proteomics with antibody-based immunoassays, whereas they 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 in terms of being antibody-free, high multiplex, 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 and high-specificity LC-SRM to effectively address the increased dynamic concentration range resulted from the addition of exogenous 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 feature and no need of 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.

## Guidelines

### 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.

### INTRODUCTION

Recent technological advances in genomics (transcriptomics) allow for comprehensive precise analysis of genome (transcriptome) in single cells [1-3]. However, single-cell proteomics technologies are lagging far behind but as equally important as genomics (transcriptomics) technologies [4-8]. Furthermore, protein abundance cannot necessarily be inferred from mRNA abundance [9], and proteome is more complex and dynamic than 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 ( $\geq 150$  proteins in a single analysis [19]), high quantitation accuracy (absolute amounts or concentrations), high specificity and reproducibility ( $\leq 10\%$  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  $\sim 670$ - $870$  proteins out of the total  $\geq 12,000$  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 downscaling sample processing volume to 2-200 nL, 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 single tube was used for single-cell processing [29], resulting in low reproducibility with a correlation coefficient of only  $\sim 0.2$ - $0.4$  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 100s of proteins in a single analysis but with relatively lower detection sensitivity [30]. Therefore, cLC-SRM is more readily to access and should have more broad 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 by absolute targeted quantification of EGFR/MAPK pathway proteins in 1-100 MCF7 or MCF10A cells along with our previously well-established SRM assays and determined 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.

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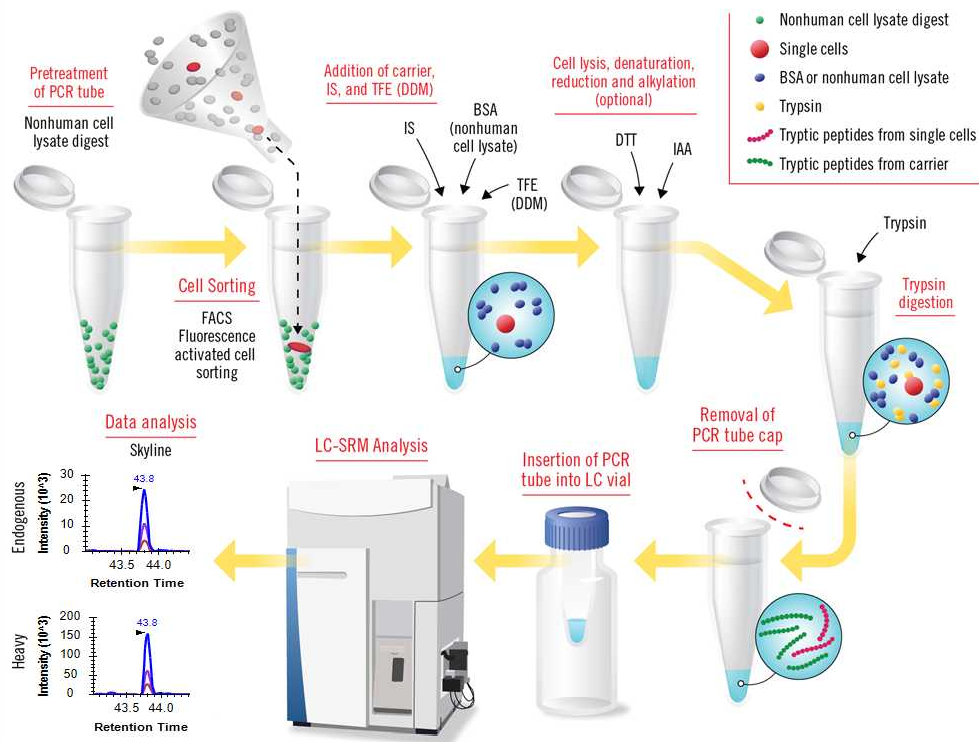
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## Before start






**Figure 1: Overview of all the 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*) is 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.


The step-by-step cLC-SRM analysis is shown in **Figure 1**. Below is the detailed protocol:

## PROCEDURE

### 1 Pretreatment of PCR tubes

- 1.1 Add  100  $\mu\text{L}$  of nonhuman (e.g., *Shewanella oneidensis*) cell lysate digests at  0.2  $\mu\text{g}/\mu\text{L}$  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 for 3 times, and then airdry PCR tubes in a fume hood.
- 1.3 Store coated PCR tubes into  4  $^{\circ}\text{C}$  freezer till further use.


### 2 FACS sorting


- 2.1 Align a fluorescence-activated cell sorter (FACS) (e.g., BD Influx flow cytometer) into a 96-well PCR plate using fluorescent beads with dyes that can be excited by 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: 1) Set the angle of the sort stream to the minimum possible to maximize the success rate so that the cells will be sorted into the bottoms of wells rather than hit the sides; 2) To aim the machine, use the test stream to 'sort' droplets of PBS buffer (e.g., 100 droplets) onto the well of empty plate to make sure that the collected droplets are on the bottom. If the droplets do not land onto 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 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  $\times g$  for 10 min at  4  $^{\circ}\text{C}$  to keep them at the bottom of the tube to avoid potential cell loss.




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

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

3.1 Add  4 µL of 25 mM  $\text{NH}_4\text{HCO}_3$  into PCR tubes containing collected cells.


3.2 Add  1 µL of 10 ng/µL bovine serum albumin (BSA) in 25 mM  $\text{NH}_4\text{HCO}_3$  into sample tubes.

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

#### Note


**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  $\text{NH}_4\text{HCO}_3$  and dispense of 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 interval for a total of 5 cycles at the amplitude at 70% 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****Note:**

TFE is a volatile solvent with boiling point of  $74\text{ }^{\circ}\text{C}$ , 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  $1500\text{ g}$  for 3 min.

**5 Reduction and alkylation (optional)**

5.1 Add  $0.6\text{ }\mu\text{L}$  of 50 mM DTT with the final concentration of 2 mM. DTT solution need to be freshly prepared each time.

**Note****Note:**

Weigh  $77.5\text{ mg}$  of DTT and then dissolve into  $1\text{ mL}$  of 25 mM  $\text{NH}_4\text{HCO}_3$  to make 500 mM DTT.  $10\text{ }\mu\text{L}$  of 500 mM DTT is then added into  $90\text{ }\mu\text{L}$  of 25 mM  $\text{NH}_4\text{HCO}_3$  to make 50 mM DTT.





5.2 Centrifuge at  $1500\text{ g}$  for 5 min and then gently mixed at 850 rpm for 3 min.

5.3 Incubate at  $56\text{ }^{\circ}\text{C}$  for 1 h with gentle shaking at  $100\text{ g}$ .

5.4 Cool samples to room temperature and centrifuge at  $1500\text{ g}$  for 3 min.

5.5 Add  $0.5\text{ }\mu\text{L}$  of 60 mM IAA to the RT-PCR tube with the final concentration of  $\sim 2\text{ mM}$ . IAA solution need to be freshly prepared each time.

**Note****Note:**

Weigh  74.3 mg of IAA and then dissolve into  1 mL of 25 mM  $\text{NH}_4\text{HCO}_3$  to make 400 mM IAA.  15  $\mu\text{L}$  of 400 mM IAA is added into  85  $\mu\text{L}$  of 25 mM  $\text{NH}_4\text{HCO}_3$  to make 60 mM IAA.

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


**Note****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 down to ~4  $\mu\text{L}$  for greatly reducing TFE volume using a Speed Vac concentrator.







**Note****Note:**

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

- 6.2 Add  9  $\mu\text{L}$  of 25 mM  $\text{NH}_4\text{HCO}_3$  and 1-3  $\mu\text{L}$  of 15 ng/ $\mu\text{L}$  trypsin for digestion with the final trypsin concentration 1-3 ng/ $\mu\text{L}$ .

**Note****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 g for 3 min and incubate overnight (~16 hours) at  37 °C .
- 6.4 Add  0.5 µL of 5% formic acid to stop enzymatic reaction.
- 6.5 Centrifuge for 1 h at  1500 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 PCR tube that is 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****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  $\mu\text{m}$  i.d.  $\times$  20 cm for standard gradient and 100  $\mu\text{m}$  i.d.  $\times$  10 cm for short gradient) to a chemically etched 20  $\mu\text{m}$  i.d. fused silica electrospray emitter via a stainless metal union.
- 8.3 Use  20  $\mu\text{L}$  sample loop for directly loading all of the sample ( $\sim 15$   $\mu\text{L}$  in total) into LC column to maximize detection sensitivity.
- 8.4 Use 0.1% formic acid in water as mobile phase A and 0.1% formic acid in 90% acetonitrile as mobile phases B for capillary RPLC separation.
- 8.5 Use the binary LC gradient at flow rates of 300 nL/min for 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 short gradient when small numbers of target proteins ( $\leq 10$ ) 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  $2,400 \pm 100$  V, a capillary offset voltage of 35 V, a skimmer offset voltage of -5 V and a capillary inlet temperature of  220  $^{\circ}\text{C}$ .
- 8.8 Obtain the other QQQ MS parameters from automatic tuning and calibration without further optimization.
- 8.9 Spike different concentrations of crude heavy peptide standards into 20-50 ng/ $\mu\text{L}$  of nonhuman cell lysate digests before LC-SRM analysis of samples to determine peptide LC retention time (RT) for building scheduled SRM method and potential interference transitions for accurate quantification.
- 8.10 Use the RT scheduled SRM mode for multiplexed quantification with the scan window of  $\geq 6$  min.

#### Note

**Note:**

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

- 8.11 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 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 having high SRM signal but without matrix interference for protein quantification and 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  $\pm 15$  s for standard gradient and  $\pm 6$  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  $S/N \geq 7$ : 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.

## DISCUSSION

- 10 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, 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 low volume (~15  $\mu$ L) 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  $\leq 10\%$  for effective digestion. To avoid drying out samples resulting in unrecoverable loss, frequently checking the sample volume (~4  $\mu$ L 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 neglectable effect on digestion efficiency. One drawback is that cysteine containing peptides cannot be selected as surrogate peptides for target proteins. To avoid Speed Vac concentrating step for TFE removal we consider replacing TFE with MS-compatible surfactants (e.g., DDM: n-Dodecyl  $\beta$ -D-maltoside) for cell lysis because frequent checking 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, single carrier protein (e.g., BSA) may not be sufficient to unbiasedly reduce surface adsorption losses for every protein. 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 be greatly beneficial to current biomedical research and systems biology.

