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Deep Proteome Coverage and Label-Free Proteomic Analysis of Low Numbers of Mammalian Cells with a Quadrupole-Ion trap-Orbitrap Mass Spectrometer

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ABSTRACT

Purpose: To improve the proteome coverage and label-free quantitation performance of limited number of mammalian cells.

Methods: Different sample preparation methods and LC-MS parameters were compared for protein identification and quantitation performance evaluation. Thermo Scientific™ Orbitrap Fusion™ Lumos™ Tribrid™ Mass Spectrometer was used for data acquisition. MS2 HCD ion trap scan was used for peptide identification and high resolution MS1 precursor was used for label free quantification. Data analysis was performed by Thermo Scientific™ Proteome Discoverer™ 2.4 with Sequest HT and MSPepSearch.

Results: RapiGest lysis buffer, lower reaction volume and higher enzyme-protein ratio could achieve higher protein and peptide IDs (i.e., deeper proteome coverage). Around 2000 proteins and 6000 peptides were identified in 40 cells using the optimized method. And around 4300 proteins and 25000 peptides were identified in 800 cells. Median CVs of protein and peptide abundances were less than 20% and 11% in lower and higher loading amount samples, respectively, and the dynamic range could reach to 5 orders of magnitudes in both lower and higher loading amount samples.

INTRODUCTION

Mass spectrometry-based proteomic experiments utilizing samples derived from a small number of cells have great potential for answering biological or clinical questions, but are accompanied by great challenges. In this study, we optimized the in-tube sample preparation method (reaction volume, lysis buffer, nucleic acid removal methods, enzyme-protein ratio) as well as some important LC-MS parameters (LC gradients, MS2 max IT) to test the proteome coverage and label-free proteomic performance of a Thermo Scientific Orbitrap Fusion Lumos instrument with low numbers of Hela cells (equivalent cell lysate). Our data demonstrated with optimized sample preparation and LC-MS methods, the instrument could achieve high sensitivity and reproducibility for the robust qualitative and quantitative proteomic analysis of limited samples.

MATERIALS AND METHODS

Sample Preparation

2.5 × 10^5 Hela cells were lysed in 500 µL different lysis buffer (0, 0.1% or 0.2% RapiGest, 5mM DTT, 50mM ammonium bicarbonate (ABC)), heat for 30min at 70 ℃ or 5min at 95 ℃ then sonication for 2 mins (no RapiGest buffer) according to references1-2. Then additional sonication or nuclease treatment was employed to remove the possible interfering nucleic acid for the RapiGest method. Resulted lysate equivalent to 2500 cells per 5 µL. Solutions equivalent to different number of cells (2500, 1000, 500, 250, 100 and 50 Hela cells per 5 µL) were subsequently prepared by diluting the lysate before Lys-C Trypsin digestion in a PCR tube. After acidified with formic acid (final concentration 6%), samples were transferred to glass insert for LC-MS analysis (Figure 1).

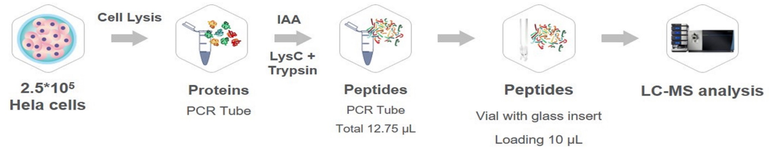
Liquid Chromatography and Mass Spectrometry

Peptides from different sample preparation methods and different equivalent cells were separated by RP-HPLC using a Dionex™ Ultimate 3000 system connected to a Thermo Scientific™ Acclaim™ PepMap™ 100 C18 column, 15 cm × 50 µm over a 90min 5-32% gradient (A: water, 0.1% formic acid; B: 80% acetonitrile, 0.1% formic acid) at 100 nL/min flow rate with 10 µL injection volume. So the final peptide loading amount was equivalent to 2000, 800, 400, 200, 80 and 40 Hela cells, respectively. The peptides were analyzed on Orbitrap Fusion Lumos Tribrid Mass Spectrometers with the following parameters, MS1 240K resolution, 250% AGC, 250ms max IT; MS2 HCD ion trap Rapid scan, 300% AGC, 50 or 80 ms max IT, Top 3 sec DDA acquisition.

Data Analysis

Spectral data files were analyzed by Proteome Discoverer 2.4 software using SEQUEST®-HT search engine or MSPepSearch spectral library search. Oxidation (+15.996 Da) used as a variable modification for methionine in addition to a static carbamidomethylation (+57.021 Da) modification for cysteine. Data was searched against a Swiss-Prot® human databases (Sequest HT) or three spectral libraries (NIST_Human_Orbitrap_HCD_20160923, NIST_Proteome Tools_Human_synthetic_HCD_20170530, Proteome Tools_HCD28_PD) (MSPepSearch) with a 1% FDR criteria for peptides and PSMs.

Figure 1. Sample preparation procedure. Solutions equivalent to different number of cells (2500, 1000, 500, 250, 100 and 50) were digested in a total 12.75 µL reaction volume. And the final peptide loading amount was 10 µL, which equivalent to 2000, 800, 400, 200, 80 and 40 Hela cells, respectively.



RESULTS

Figure 2. Different maximum MS2 injection times were then optimized to get higher IDs (60min gradient). Results showed 50ms was the optima MS2 Max IT for both 2000 (A) and 400 (B) cells samples. The rest samples could be done in the same manner.

Loading amount	Optimized MS2 Max IT
80-2000 cells	50ms
40 cells	80ms

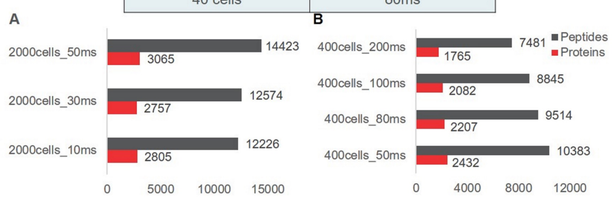


Figure 3. The effect of different sample preparation methods (lysis buffer (A), Nucleic acid removal and enzyme-protein ratio (B)) on the IDs of proteins and peptides (2000 cells, 90min gradients). In the comparison of lysis buffer, A549 cells were employed, Hela cells were used for the rest. 0.1% RapiGest, higher enzyme-protein ratio could give more IDs of both proteins and peptides. But different nucleic acid removal methods seemed to have small impact on the identification results and will be further discussed in the following study.

A

Sample preparation	Method
Cell Lysis buffer	L1: 0.1% RapiGest L2: 0.2% RapiGest L3: no RapiGest, 50mM ABC

B

Sample preparation	Method
Nucleic acid removal	N1: No sonication, No nuclease N2: Nuclease N3: Sonication
Enzyme：protein	Low: 4ng per 2000 cells High: 200ng per 2000 cells

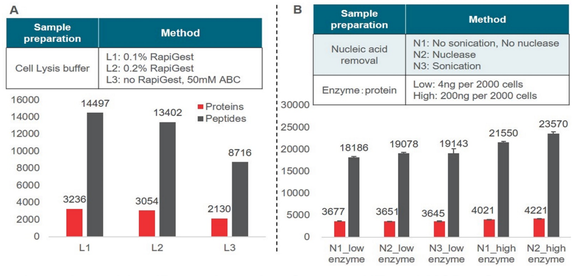


Figure 4. Protein and peptides ID results using Sequest HT and MSPepSearch, respectively (take 800 cells, 400 cells, and 200 cells for example). Compared to Sequest HT, MSPepSearch could results in more proteins and peptides (ID number of proteins and peptides were increased by 7% and 13-18%) and was used in the final result.

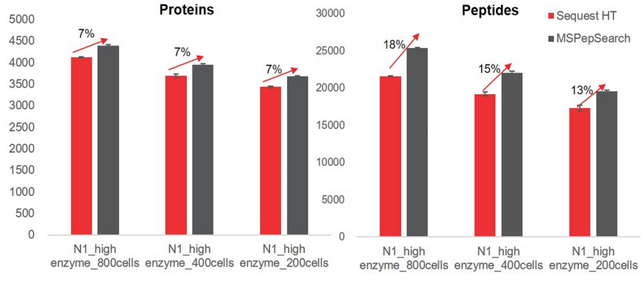


Figure 5. Protein and peptide identification results from 40cells to 800cells. Around 2000 proteins and 6000 peptides were identified in 40 cells using the optimized method. And around 4300 proteins and 25000 peptides were identified in 800 cells.

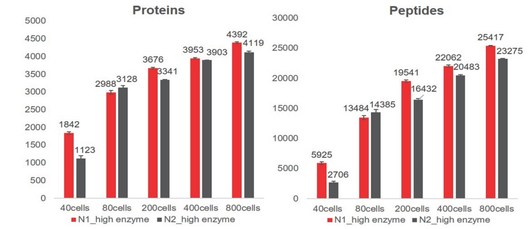
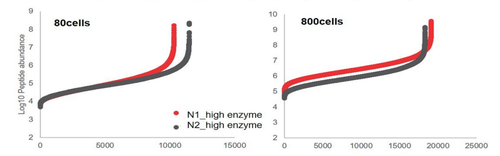


Figure 6. Quantification performance evaluation. A, protein and peptide abundance median CV of three technical replicates. B, dynamic range of peptide abundance in 80cells and 800 cells. Results showed over 5 orders of magnitudes of dynamic range.

Abundance median CV						
Proteins	N1_high enzyme	N2_high enzyme	Peptides	N1_high enzyme	N2_high enzyme
40cells	17.65%	16.17%	40cells	18.88%	16.62%
80cells	16.30%	11.11%	80cells	17.60%	13.88%
200cells	8.85%	15.46%	200cells	10.32%	17.35%
400cells	10.03%	9.77%	400cells	10.45%	11.20%
800cells	8.62%	8.33%	800cells	9.56%	9.29%



CONCLUSIONS

- 0.1% RapiGest lysis buffer, lower 12.75 µL reaction volume and higher enzyme-protein ratio (200ng per 2000 cells) combined with lower ID column (50µm) and lower flow rate (100 nL/min), compared to routine proteomic work could increase the sensitivity when limited sample was involved. With high reproducibility, this robust workflow may expand its application to other similar situations with appropriate modification.
- Compared to search engine based method for proteomic identification, spectral library based method showed superior performance, results in more protein and peptide IDs, which makes it an alternative way for limited sample and routine proteomic work.
- The Orbitrap Fusion Lumos instrument showed deep proteome coverage and good label free quantitative performance for low numbers of Hela cells (down to 40 cells with around 2000 protein IDs, low median CVs of protein and peptide abundance, 5 orders of magnitudes of dynamic range) without using complicated microfluid-based sample preparation methods.

REFERENCES

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2. Schoof EM, Rapin N, Savickas S, Gentil C, Lechman E, Haile JS, Keller U, Dick JE, Porse BT. A Quantitative Single-Cell Proteomics Approach to Characterize an Acute Myeloid Leukemia Hierarchy. bioRxiv, http://dx.doi.org/10.1101/745679.

TRADEMARKS/LICENSING

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