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pH/Acetonitrile-Gradient Reversed-Phase Fractionation of Enriched Hyper-Citrullinated Library in Combination with LC–MS/MS Analysis for Confident Identification of Citrullinated Peptides

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Abstract

Citrullination, the Ca^{2+}-driven enzymatic conversion of arginine residues to citrulline, is a posttranslational modification, implicated in several physiological and pathological processes. Several methods to detect citrullinated proteins have been developed, including color development reagent, fluorescence, phenylglyoxal, and antibody-based methods. These methods yet suffer from limitations in sensitivity, specificity, or citrullinated site determination. Mass spectrometry (MS)-based proteomic analysis has emerged as a promising method to resolve these problems. However, due to low abundance of citrullinated proteins and similar MS features to deamidation of asparagine and glutamine, confident identification of citrullinated proteome is challenging. Here, we present a systematic approach to identify a compendium of steps to enhance the number of detected citrullinated residue and implement diagnostic MS feature that allow the confidence of MS-based identifications. Our method is based on the concept of generation of hyper-citrullinated library with high-pH reversed-phase peptide fractionation that allows to enrich in low abundance citrullinated peptides and amplify the effect of charge loss upon citrullination. Application of our approach to complex global citrullino-proteome datasets demonstrates the confident assessment of citrullinated peptides, thereby enhancing the size and functional interpretation of citrullinated proteomes.

Keywords

Citrullination; Deamidation; Retention time; Hyper-citrullinated peptide library; Mass spectrometry; pH reversed-phase peptide fractionation

1 Introduction

Citrullination or deimination is a process catalyzed by specific enzymes called peptidylarginine deiminase (PAD) in which arginine amino acid is converted into citrulline amino acid [1]. In this reaction, hydrolysis of the ketimine group (double bond NH) of the arginine side chain results in the formation of citrulline, ketone group (double bond O), and ammonia as a side product [2, 3]. This irreversible conversion is accompanied by the loss of the arginine positive charge, isoelectric point of arginine is 11.41, at physiological pH, whereas the ureido group of the citrulline has isoelectric point 5.91 [4, 5]. The exact role of the modification remains largely unknown, but it is believed to substantially affect the binding and unfolding properties of the protein, resulting in the alteration of protein function and half-life [6, 7]. To date, it has been confirmed that arginine residues of dozens of proteins can undergo citrullination. The substrates could be enolase, vimentin, keratin, myelin, filaggrin, histones, serine protease inhibitors, proteases, and metabolic enzymes [8–11]. Moreover, citrullination is known to play a role in the disease etiology of the joint disease rheumatoid arthritis (RA) [12], and many of the citrulline residues were recognized as trigger for immune response to generate autoantibodies against citrullinated epitopes [13, 14].

Despite a lot of emerging biology, protein citrullination is far less well studied compared to other posttranslational modification (PTMs). This can in part be attributed to a lack of robust biochemical enrichment and detection methods. Currently, apart from immunodetection techniques, relying on the specificity and sensitivity of the antibodies, citrullination could only be characterized at the molecular level using liquid chromatographic tandem mass spectrometric methods (MS). The MS-based analysis of citrulline-modified residue relies on the detection of the mass increment of 0.9840 Da compared with the unmodified arginine residue. However, the method has its limitations due to lack of specificity, as the same, 0.9840 Da mass difference commonly occurred as deamidation of glutamine or asparagine residues leading to serious ambiguity in database searching and suffer from high false-positive rates [15]. To improve false-positive rates, the latter method identifies characteristic ions for citrullination produced by MS/MS analysis [16], called diagnostic ions. Comparison of MS/MS spectra for citrullinated and unmodified synthetic peptides revealed immonium ions (130.097 m/z) of citrulline and ions produced from neutral loss (NL) of isocyanic acid (HNCO, 43.0058 Da) as diagnostic ions for citrullination. Whether diagnostic ions are enough to improve the accuracy has been rarely investigated due to the required of the manual validation of citrullinated peptides [17]. As an alternative approach, Larsen et al. used reversed-phase (RP) modality to detect shift in retention time for citrullinated peptides compared with the corresponding unmodified peptides. The approach described before [18, 19] represents the chromatographic feature for detection of citrullinated peptides eluted from C18 reverse phase, based on loss of positive charge and increase hydrophobicity. In complex samples, the method is limited to the situation non-modified peptides analog has been detected in the same run.

To overcome this limitation, Fert-Bober et al. induced enzymatic citrullination with PADs cocktail followed by Lys-C digestion to generate hyper-citrullinated peptide library followed by reproducible and straightforward fractionation method. This protocol describes the

step-by-step library generation workflow required to confidently identify and validate citrullinated peptides using: (1) citrullinated peptide enrichment, (2) high-pH fractionation, (3) LC–MS/MS analysis, and (4) data analysis of the obtained fractions (Figs. 1, 2, and 3). In our study, we also present a novel data analysis tool called CitFinder that utilizes physiochemical properties such as delta retention time shift (ΔRT) and neutral loss of isocyanic acid to identify and validate citrullinated peptide signatures. The simple fractionation system together with peptide retention time measurement and software algorithms for automated large citrullinated proteome search increases accuracy and reliable identification of modified proteins/peptides.

2 Materials

Prepare solutions using ultrapure water and store all solutions and reagents at room temperature (unless indicated otherwise). All solutions and reagents are enough to process 20 samples.

2.1 Cell/Tissue Lysis

1. 1 M Tris–HCl (pH 7.6): mix and dissolve 12.11 g Tris in ultrapure water to a final volume of 100 mL. Adjust the pH to 7.6 with 10 M HCl using pH meter.
2. 20% sodium dodecyl sulfate (SDS): mix and dissolve 20 g SDS in 100 mL of ultrapure water (*see* Note 1).
3. 1 M ditiotreitol (DTT): mix and dissolve 154.2 mg DTT in 1 mL of ultrapure water (*see* Note 2).
4. Cell/tissue lysis buffer: 2% SDS, 50 mM DTT in 100 mM Tris–HCl (pH 7.6): mix following solutions in ultrapure water to a final volume of 20 mL: 2 mL of 20% SDS, 1 mL of 1 M DTT and 2 mL of 1 M Tris–HCl (pH 7.6)—has to be freshly prepared.
5. 1× PBS (pH 7.4).
6. Liquid nitrogen.
7. 1.5-mL low binding tubes.
8. Ceramic mortar and pestle.
9. Sonicator.
10. ThermoMixer.
11. Centrifuge.

2.2 Protein Quantification

1. The Pierce 6600-nm Protein Assay Kit (Thermo Fisher Scientific) contains Pierce 660 nm Protein Assay Reagent (450 mL) and Pre-diluted Protein Assay Standards: bovine serum albumin (BSA) (set 7 × 3.5 mL at a specific concentration from 125 to 2000 μg/mL in 0.9% saline and 0.05% sodium azide, store at 4 °C).

2. Ionic Detergent Compatibility Reagent (IDCR, Thermo Fisher Scientific): mix and dissolve 1 g in 20 mL of the Pierce 660 nm Protein Assay Reagent before use (store up to 1 week at 4 °C).
3. 96-well plate.
4. Spectrophotometer.

2.3 Filter-Aided Sample Preparation (FASP) Reagents

1. 1 M Tris–HCl (pH 8.5): mix and dissolve 12.11 g Tris in ultrapure water to a final volume of 100 mL. Adjust the pH to 8.5 with 10 M HCl using pH meter.
2. Urea buffer—8 M urea in 100 mM Tris–HCl (pH 8.5): mix and dissolve 9.6 g urea in 2 mL of 1 M Tris–HCl (pH 8.5) and 18 mL of ultrapure water (*see* Note 3).
3. Digestion (DB) buffer—50 mM Tris–HCl (pH 8.5): mix 1 mL of 1 M Tris–HCl (pH 8.5) with 19 mL ultrapure water.
4. Lysyl Endopeptidase®, Mass Spectrometry Grade (Lys-C) (FUJIFILM Wako Pure Chemical Corporation): dissolve 20 μg Lys-C in 40 μL DB buffer to make 0.5 μg/μL concentration of Lys-C—prepare directly before use (store at −70 °C).
5. 1 M iodoacetamide (IAA): mix and dissolve 185 mg IAA in ultrapure water to a final volume of 1 mL (*see* Note 4).
6. 0.5 M NaCl: mix and dissolve 292.3 mg NaCl in ultrapure water to a final volume of 10 mL.
7. Microcon-30 kDa Centrifugal Filter Unit with Ultracel-30 membrane—0.5 mL centrifugal filters (Merck Millipore).
8. Conical tubes (50 mL).
9. Closed plastic box with tube holder and sponge inside.
10. Incubator.
11. Centrifuge.

2.4 Deamidation Reaction

1. 1 M $CaCl_2$: mix and dissolve 1.11 g $CaCl_2$ in 10 mL of ultrapure water.
2. Deimidation buffer—5 mM $CaCl_2$, 0.5 mM DTT, 100 mM Tris–HCl (pH 7.6): mix following solutions in ultrapure water to a final volume of 2000 μL: 10 μL of 1 M $CaCl_2$, 1 μL of 1 M DTT, 200 μL of 1 M Tris–HCl (pH 7.6) (*see* Note 5).
3. PAD Cocktail, Active (SignalChem)—the five full-length recombined PAD proteins (1, 2, 3, 4, and 6): enzyme concentration 0.5 μg/μL (store at −70 °C).

2.5 Desalting

1. Methanol (mass spectrometry grade).

2. 0.1% formic acid (FA) in water (mass spectrometry grade).
3. 0.1% FA in 50% acetonitrile (ACN) (mass spectrometry grade).
4. 10% trifluoroacetic acid (TFA): mix 1 mL of TFA with 10 mL of ultrapure water.
5. Oasis HLB 96-well μElution Plate, 2 mg Sorbent per Well, 30 μm (Waters).
6. 1.5-mL low binding tubes.
7. 96-Well Plate Vacuum Manifold.
8. Vacuum concentrator.

2.6 High-pH Reversed-Phase Fractionation

1. Pierce™ High-pH Reversed-Phase Peptide Fractionation Kit (Thermo Fisher Scientific): contains Reversed-Phase Fractionation Spin Columns (12 columns containing 20 mg of resin in a 1:1 water/DMSO slurry) and triethylamine (0.1% in water, 100 mL).
2. 0.1% trifluoroacetic acid (TFA): mix 10 μL of TFA with 10 mL of ultrapure water.
3. Acetonitrile (ACN) (mass spectrometry grade).
4. 2-mL low binding tubes.
5. Microcentrifuge.
6. Vacuum concentrator.

2.7 Mass Spectrometry Sample Preparation

1. 0.1% formic acid (FA) in water (mass spectrometry grade).
2. iRT Kit (BIOGNOSYS): to prepare 10× iRT Standard add 50 μL of Dissolution Buffer to the iRT Standard tube and mix well according to the manufacturer's instructions [20] (*see* Note 6).
3. Solvent A 100% water in 0.1% formic acid, solvent B 100% acetonitrile in 0.1% formic acid.
4. 0.3 × 10 mm trap cartridge packed with ChromXP C18CL 120A.
5. 0.3 × 150 cm column packed with ChromXP C18CL 120A.
6. TripleTOF 6600 system (SCIEX) (*see* Note 14) couple with DuoSprayTM Source with a 25 μm I.D. hybrid electrode (SCIEX).

2.8 Data Analysis Software and Setup

1. Computer: Operating system with Windows or Linux with ≥4 GB of RAM.
2. Python version 2.7+ with Anaconda: Install Anaconda from https://www.anaconda.com/products/individual, which includes a python interpreter and all the required python libraries such as pip, numpy, pandas, and cython. To

check if installation is successful, run python --version on the command line to show the version of python installed (*see* Note 7).

3. msproteomicstools python package: Using the command line, run C:/Anaconda/Scripts/pip.exe install msproteomicstools to install msproteomicstools package.

4. Git: Install Git from https://git-scm.com/downloads for the appropriate operating system. To check if installation is successful, run git --version on the command line to show the version of Git installed.

5. CitFinder python script: On the command line, navigate to the directory where you want to install CitFinder and run the command git clone https://github.com/Citrullinome/CitFinder.git. This will set up a folder called CitFinder containing the executable python script CitFinder.py as well as a sub-folder called Example. This sub-folder contains an example dataset with the necessary input files to demonstrate the use of CitFinder, along with its generated output files (*see* Subheading 3.11).

3 Methods

3.1 Cell/Tissue Lysis

1. To prepare hyper-citrullinated library of cells, grow selected cells in 10-cm dishes to a confluence of 80–90%.

2. After cells reach 80–90% of confluence, wash dishes with 5 mL of 1× PBS (pH 7.4).

3. Add 300 μL of lysis buffer: 2% SDS, 50 mM DTT in 100 mM Tris–HCl (pH 7.6) and distribute thoroughly to cover the entire area of dish (*see* Note 8).

4. Freeze the dishes at −70 °C for 15 min (*see* Note 9).

5. After thawing scrape the dish with broken 200 μL pipette tip and transfer lysed solution to the low binding tube.

6. Vortex and sonicate for 10 min.

7. Boil samples at 95 °C for 10 min using ThermoMixer.

8. Centrifuge for 15 min at 14,000 × *g* and then transfer the supernatant to the new 1.5-mL tube.

9. Freeze at −70 °C or proceed to the next step.

10. To prepare hyper-citrullinated library of tissue crash the selected piece of tissue in liquid nitrogen using pre-cooled at −70 °C ceramic mortar and pestle.

11. Weigh the powdered tissue and dissolve in lysis buffer: for each 10 mg of tissue add 100 μL of lysis buffer.

12. Proceed to the **steps 6–9**.

3.2 Protein Quantification

1. Use the Pierce 660 nm Protein Assay Kit to measure protein concentration in the samples according to the manufacturer's instructions (*see* Note 10).
2. Briefly, dilute the samples 1:5 in ultrapure water and transfer 10 μL of each diluted sample into a microplate well.
3. Prepare a standard curve in 96-well plate: add 10 μL of each pre-diluted standard and blank into a microplate well.
4. Add 150 μL of the Protein Assay Reagent with Ionic Detergent Compatibility Reagent to each well.
5. Mix thoroughly and incubate at room temperature for 5 min.
6. Read the absorbance at 660 nm.

3.3 FASP Digestion with Lys-C and Deimidation Reaction on Filters

1. To digest proteins and preserve citrullination residues use filter-aided sample preparation (FASP) method with Lys-C as a digestion enzyme [21, 22] (*see* Note 11).
2. Process each sample in two replicates: sample treated with PAD cocktail (hyper-citrullinated sample, marked as PAD+) and non-treated sample without PAD cocktail (unmodified sample, marked as PAD-).
3. Place the Microcon-30 centrifugal filter into 1.5-mL tube, add 250 μL of urea buffer with 100 μg of protein, mix well, and centrifuge for 20 min at 10,000 × *g* at room temperature (*see* Note 12).
4. Add 250 μL of urea buffer, centrifuge for 20 min at 10,000 × *g* at room temperature, and then pour the filtered solution.
5. Add 250 μL of urea buffer, centrifuge for 20 min at 10,000 × *g* at room temperature.
6. Add 200 μL of urea buffer, centrifuge for 20 min at 10,000 × *g* at room temperature, pour the filtered solution.
7. Add 100 μL of 0.055 M IAA (to prepare 0.055 M IAA mix 122 μL of 1 M IAA and 2078 μL of urea buffer) to the filter, incubate for 20 min in the dark at room temperature, and then centrifuge for 20 min at 10,000 × *g* at room temperature.
8. Add 200 μL of urea buffer, centrifuge for 20 min at 10,000 × *g* at room temperature.
9. Add 200 μL of DB buffer, centrifuge for 20 min at 10,000 × *g* at room temperature.
10. Add 200 μL of DB buffer, centrifuge for 20 min at 10,000 × *g* at room temperature, pour the filtered solution.

11. Add 100 μL of deimidation buffer to the filter. To PAD+ samples, add 10 μL of PAD cocktail in a ratio of 1:20 (enzyme to protein ratio). To PAD-samples, add 10 μL of ultrapure water. Mix thoroughly.
12. Incubate for 2 h at 37 °C.
13. Centrifuge for 20 min at 10,000 × *g* at room temperature.
14. Add 200 μL of DB buffer, centrifuge for 20 min at 10,000 × *g* at room temperature.
15. Add 200 μL of DB buffer, centrifuge for 20 min at 10,000 × *g* at room temperature, pour the filtered solution, and transfer the filter to the new collection tube.
16. Add 6 μL (3 μg) Lys-C in 60 μL of DB buffer to the filter (stock: 0.5 μg/μL in DB buffer, enzyme to protein ratio 1:35), mix thoroughly, and incubate overnight at 37 °C in closed plastic box with wet sponge inside (*see* Note 13).
17. Elute for 10 min at 10,000 × *g* at room temperature, collect the filtered solution.
18. Add 125 μL of DB buffer, centrifuge for 10 min at 10,000 × *g* at room temperature, collect the filtered solution.
19. Add 100 μL of DB buffer, centrifuge for 10 min at 10,000 × *g* at room temperature, collect the filtered solution.
20. Add 50 μL of 0.5 M NaCl, centrifuge for 10 min at 10,000 × *g* at room temperature, collect the filtered solution.
21. Store at −70 °C or proceed to the next step.

3.4 Desalting

1. Place the Oasis HLB 96-well μElution Plate into 96-Well Plate Vacuum Manifold.
2. Activate C18 reversed-phase sorbent with 400 μL of methanol: add 400 μL of methanol to each well in 96-well plate, turn on the vacuum to pass the solvent through sorbent.
3. Repeat the **step 2**.
4. Add 400 μL of 0.1% FA, turn on the vacuum.
5. Repeat the **step 4**.
6. Acidify samples after FASP with 10 μL of 10% TFA (*see* Note 14).
7. Load digested samples into 96-well plate, turn on the vacuum, and collect passed samples in 96-well collection plate.
8. Repeat the **step 7**—load again collected samples.
9. Wash with 400 μL of 0.1% FA.
10. Repeat the **step 9**.

11. Elute the peptides into 96-well elution plate with 200 μL of 0.1% FA in 50% ACN.
12. Repeat the **step 11** twice, collect eluted peptides, and transfer to the new low binding tubes.
13. Dry desalted samples in vacuum concentrator.
14. Store at −70 °C or proceed to the next step.

3.5 High-pH Reversed-Phase Fractionation and Mass Spectrometry Sample Preparation

1. To fractionate desalted samples, use Pierce™ High-pH Reversed-Phase Peptide Fractionation Kit (Thermo Fisher Scientific) according to the manufacturer's instructions with the following modifications: fractionate each sample into 5 fractions (instead of 8) with 7.5%, 10%, 12.5%, 15%, and 17.5% ACN in elution solution (fractions from 2 to 6, respectively) (*see* Note 15).
2. Dry fractionated samples in vacuum concentrator.
3. Store at −70 °C until samples are ready for LC-MS runs.
4. Dissolve each fraction in 10 μL of 0.1% FA.
5. Add 1 μL of 10× iRT Standard and load in a vial dedicated to LC-MS.

3.6 Liquid Chromatography

1. To fractionate LysC digested peptides, use NanoLCTM 425 System (Sciex) operating in trap elute mode at microflow.
2. Load 4 μL of samples onto a 0.3 × 10 mm trap cartage, wash with the mobile phase A for 3 min at 10 μL/min.
3. Switch the trap valve to elute the samples off the trap through a 0.3 × 150 cm column. Use 120 min gradient (3–35% solvent B, accordingly at 5 μL/min (Total run time 180 min).

3.7 Mass Spectrometry

1. To perform MS analysis, use a TripleTOF 6600 system (SCIEX) (*see* Note 16) couple with DuoSprayTM Source with a 25 μm I.D. hybrid electrode (SCIEX).
2. Use the following source parameters, spay voltage 5500 V, GS1 of 14, GS2 of 15, curtain gas of 25, heater temperature of 100, and declustering potential of 80 V.
3. Set up each cycle for 250 ms of TOF MS follow by 30 MS/MS scans at 100 ms each. Total cycle time is 3.3 s.
4. Set up collision energy for 5.
5. Set up the MS range between 400 and 1250 m/z (*see* Note 17).

3.8 Data Preparation

1. To identify citrullinated peptides, a hyper-citrullinated spectral library generated through SpectraST (*see* Note 18) is required by CitFinder to analyze modified–unmodified peptide pairs for physicochemical properties such as ΔRT shift, charge state, and neutral loss (*see* Note 19).
2. The protein sequence database file used during the database search is required by CitFinder to determine the position of the citrullinated sites.
3. An optional step includes validation of the chromatographic peak groups using Skyline [23, 24]. To accomplish this, Cit-Finder must be provided a Skyline exported report containing the fields Replicate Name, Peptide Modified Sequence, Precursor MZ, Precursor Charge, Isotope Dot Product, Best Retention Time, Total Area, Average Mass Error PPM, and Max Full Width at Half Maximum (FWHM) (*see* Note 20).

3.9 CitFinder: Identification of Citrullinated Peptides

1. Navigate to the CitFinder folder by running cd CitFinder on the command line. The included python script, CitFinder. py, requires customized command line options for execution.
2. Provide the spectral library file Cit_Mouse_Organs_SpecLib.splib as the input for the option -i.
3. Provide the protein database file UP_Mouse_Rev_Canonical_20180228_DECOY_iRT.fasta for the option -f.
4. Specify the modification nomenclature R[157] for the option -m to denote the citrullinated site (*see* Note 21).
5. Specify a comma-separated list of file-specific keywords to calculate PSM counts for each sample group Heart,Lung,Liver,Muscle,Kidney,Brain using the option -g.
6. Specify an arbitrary output file name in csv format Cit_Mouse_Organs_SpecLib_CitFinder.csv for the option -o.
7. Based on the **steps 1–6**, execute the final command line (*see* Note 22) as shown below:

```
python CitFinder.py -i Example/Cit_Mouse_Organs_-SpecLib.splib
-f Example/UP_Mouse_Rev_Canonical_20180228_DECOY_iRT.fasta -m
R[157] -g Heart,Lung,Liver,Muscle,Kidney, Brain -o Example/
Cit_Mouse_Organs_SpecLib_CitFinder.csv
```

3.10 CitFinder: Validation of Citrullinated Peptides

1. Follow **steps 1–6** as shown in Subheading 3.9.
2. Specify an arbitrary output file name in csv format Cit_Mouse_Organs_SpecLib_Skyline_CitFinder.csv for the option -o.

3. Provide the Skyline exported report Cit_Mouse_Organs_-SpecLib_Skyline.csv to categorize chromatographic peaks as “Good”, “Okay” and “Bad” using the option -s.

4. Based on **steps 1–3**, execute the final command line as shown below:

```
python CitFinder.py -i Example/Cit_Mouse_Organs_SpecLib.
splib -f Example/UP_Mouse_Rev_Canonical_20180228_DECOY_iRT.
fasta -m R[157] -g Heart,Lung,Liver,Muscle,Kidney,Brain
-o Example/Cit_Mouse_Organs_SpecLib_Skyline_CitFinder.csv -s Example/
Cit_Mouse_Organs_SpecLib_Skyline.csv
```

3.11 Anticipated Results

1. The CitFinder generated report characterizes each representative peptide spectrum match to its corresponding modified–unmodified peptide pair.

2. Some of the columns generated by the report include: Protein name, Modified peptide, Unmodified peptide, Citrullinated Site, Retention Time Shift, Charge Shift, Total Neutral Loss Ions (*see* Table 1).

3. This tool also provides an option to include PSM counts for each sample group across the total spectra. Additionally, CitFinder also categorizes chromatographic peak groups into “Good,” “Okay,” and “Bad” to aid in the validation of the citrullinated peptides (*see* Fig. 4 and Note 23).

4 Notes

1. Concentrated solutions of SDS tend to precipitate even at room temperature. Warm it up a little to dissolve precipitated particles.

2. Aliquot freshly prepared 1 M DTT and store at −20 °C for further use.

3. Urea buffer: 8 M urea in 100 mM Tris–HCl (pH 8.5) has to be freshly prepared before use. To quickly dissolve urea in 100 mM Tris–HCl, put it into a warm water bath.

4. Aliquot freshly prepared 1 M IAA and store at −20 °C for further use. Protect from the light.

5. Make sure to measure the pH of deimidation buffer. Optimal pH for PAD reaction is around 7–8. Deimidation buffer should contain a small amount of DTT to reduce disulfide bonds and make protein structure more accessible to the PAD enzymes.

6. iRT alignment is a critical step in this workflow because the identification of citrullinated peptides relies on the ΔRT shift between these peptides. Hence it is advisable to use internal retention time standards for increased accuracy.

7. Installation of Anaconda is not required if python version 2.7+ has already been installed along with the required python libraries. In this case, proceed to

installation of msproteomicstools python package by running the command: pip install msproteomicstools from the command line.

8. We use 2% SDS in our cell/tissue lysis buffer to completely extract proteins as SDS helps to solubilize and denaturate proteins. Addition of DTT reduces disulfide bonds, and then this step can be later omitted during digestion of proteins.

9. Freezing cell culture dishes covered by lysis buffer helps to destroy and lysate cells.

10. We use the Pierce 660 nm Protein Assay Kit to measure protein concentration because of the high amount of DTT (50 mM) in our lysis buffer. Standard BCA Protein Assay Kits are only compatible with 1 mM DTT. To make the Pierce 660 nm Protein Assay Kit compatible with 2% SDS, Ionic Detergent Compatibility Reagent (IDCR) should be added to the Pierce 660 nm Protein Assay Reagent.

11. We choose filter-aided sample preparation (FASP) method to digest proteins, because this method removes undigested proteins and keeps only clean peptides as well as allows for buffer exchange. Lys-C was used as a digestion enzyme instead of commonly used trypsin in order to preserve citrullinated residues. Trypsin cuts peptides at the carboxyl side of lysine or arginine, contrary to Lys-C, which cuts purely at the carboxyl side of lysine.

12. The more concentrated the sample, the better. The ratio of protein to urea buffer loaded in the filter should not exceed 1:10. If the amount of protein loaded in the filter is greater than 25 μL, load only 25 μL of sample in urea buffer and repeat multiple times.

13. For overnight digestion with Lys-C, place samples in closed plastic box with sample holder. Put inside the wet sponge to prevent the evaporation of samples.

14. Before loading samples onto C18 reverse phase sorbent make sure to acidify samples with 10% TFA and check the pH. pH of the samples should be lower than 3 to keep peptides bound to the sorbent.

15. We use Pierce™ High-pH Reversed-Phase Peptide Fractionation Kit to increase protein and citrullination sites identifications from LC-MS analysis.

16. The digested samples can be analyzed by LC–MS/MS using an Ultimate 3000 LC and an Orbitrap Lumos Fusion MS (Thermo Fisher Scientific, Waltham, MA, USA). An M3 multinozzle emitter (Newomics, Berkeley CA, USA) is used to electrospray eluting peptides into the mass spectrometer at 3.9 kV with sheath gas set to 20 (arbitrary units). Peptides are loaded onto a Luna Omega trapping column (300 μm I. D. × 2 cm, Polar C18 3 μm, 100 Å, Phenomenex) and separate over a 60-min reversed phase gradient at 8 μL/min on a Luna Omega analytical column (300 μm I.D. × 15 cm, Polar C18 3.0 μm, 100 Å). 10 μL of sample is collected by the autosampler in a 20 μL loop, and the loading pump pushes the peptides into the trapping column in 0.1% aqueous formic acid at 15 μL/min and desalt for 3 min. Then the trapping column is connected to the

analytical pump and column via a switching valve and the analytical gradient starts at 5% mobile phase B (0.1% formic acid in acetonitrile) and 99% mobile phase A (0.1% formic acid in water). The mobile phase B composition linearly increases to 30% at 45 min, then increases to 40% at 50 min, and finally linearly increases to 45% B at 60 min. After each analytical run, the columns are flushed with 95% mobile phase B for 7 min and equilibrated back to 2% B in 3 min (10 min total). The trapping and analytical columns are kept at 50 °C using the Ultimate 3000 column oven.

17. Mass spectrometry data is acquired using a data-independent method (DIA). The precursor MS1 scans are acquired at 120 K resolution and span the 400–1000 m/z range, maximum injection time is set to 50 ms and AGC target set to 400,000. After each MS1 scan, the entire 400–1000 m/z range is fragmented by HCD with 30% normalized energy in non-overlapping 15 m/z windows (40 DIA scans following each MS1 scan). The DIA scans are acquired at 15 K resolution with AGC set to 500,000 and maximum injection time of 30 ms.

18. We created the hyper-citrullinated spectral library using the following steps: (1) mass spectrometry data were acquired and converted to open-source format [25, 26]. (2) The fragment ion spectra were searched against a protein sequence database to identify PSMs [27–29]. (3) PSMs were statistically scored [30, 31] and converted into a spectral library [32, 33], which was then used as an input for CitFinder. (4) After the assessment of the citrullinated peptides using CitFinder, the retention times within the spectral library were normalized into iRT space followed by consensus library generation. (5) Finally, the resulting assay library can further be used as an input for all currently available SWATH analysis software tools [34].

19. It is recommended to use a longer gradient (for example, 120 min) for better differentiation between citrullination of arginine (R) and deamidation of asparagine (N) and glutamine (Q) based on the more pronounced ΔRT shift.

20. Prior to the Skyline validation step in CitFinder, ensure that the appropriate transition settings for precursor charges, ion type, isotope peaks, precursor mass range, and instrument settings are used. Similarly, verify the peptide settings for digestion enzyme, missed cleavages, background proteome, peptide length, and modifications of interest.

21. We provide R[157] to the command line option -m in CitFinder to indicate the nomenclature of the modified site as seen in the spectral library file. This will vary depending on how the modification is denoted in the spectral library file and must be adjusted accordingly.

22. By default, CitFinder filters out any citrullinated peptides with ΔRT shift >5 min. This filter can be turned off by passing -r False command line option. This is recommended when the complete list of citrullinated peptides is desired and/or shorter gradients are used.

23. We determined suitable peak group thresholds for validation of citrullinated peptides using Skyline as follows: Chromatographic Peak Groups with Confirmed Peptide Identity, Isotope Dot Product ≥0.9, Retention Time Drift ≤ ±0.2, Total Area ≥ 100,000, Total Area/FWHM ≥1,000,000, and Average Mass Error PPM ≤ ±5 were defined as "Good." Chromatographic Peak Groups with Confirmed Peptide Identity and meeting four of the following five quality measures, Isotope Dot Product ≥0.7, Retention Time Drift ≤ ±0.4, Total Area ≥ 10,000, Total Area/FWHM ≥ 100,000, and Average Mass Error PPM ≤ ±10, were defined as "Okay." All other Peak Groups that did not meet the above thresholds or had poor peak quality measures such as Isotope Dot Product <0.7 or Retention Time Drift ≥ ±1 were automatically flagged as "Bad."

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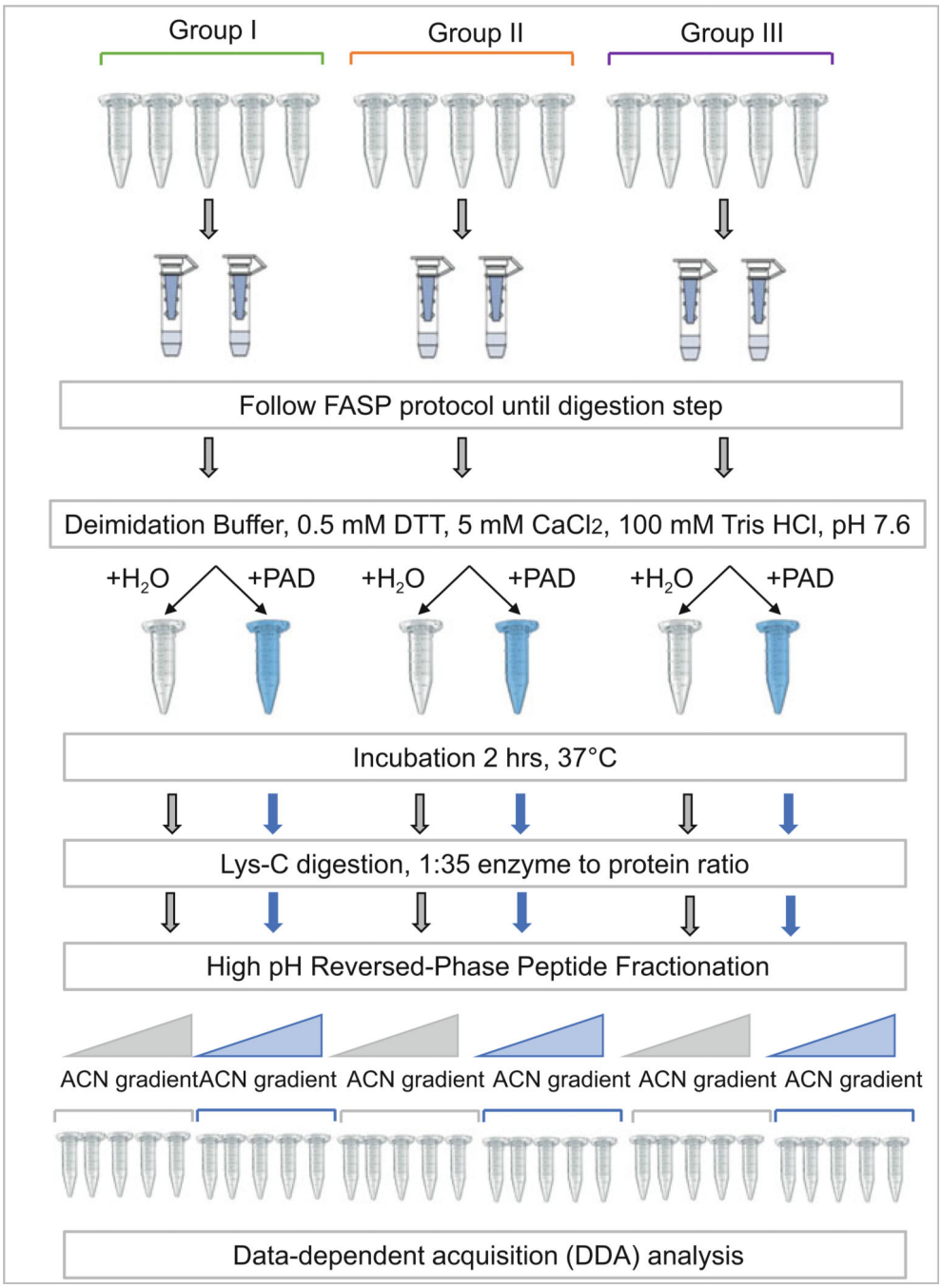

Fig. 1.

Workflow to generate hyper-citrullinated peptide library. Proteins in each individual sample were pooled (comprised of equal portions of individual samples) and divided into two samples. Following FASP steps just after IAA treatment, samples were conditioned with deamidation buffer to allow citrullination by incubation with PADs. Following citrullination and washing steps, samples were digested, and fractionated using two types of high-pH fractionation methods. Every prepared sample was analyzed on a Q Exactive mass spectrometer

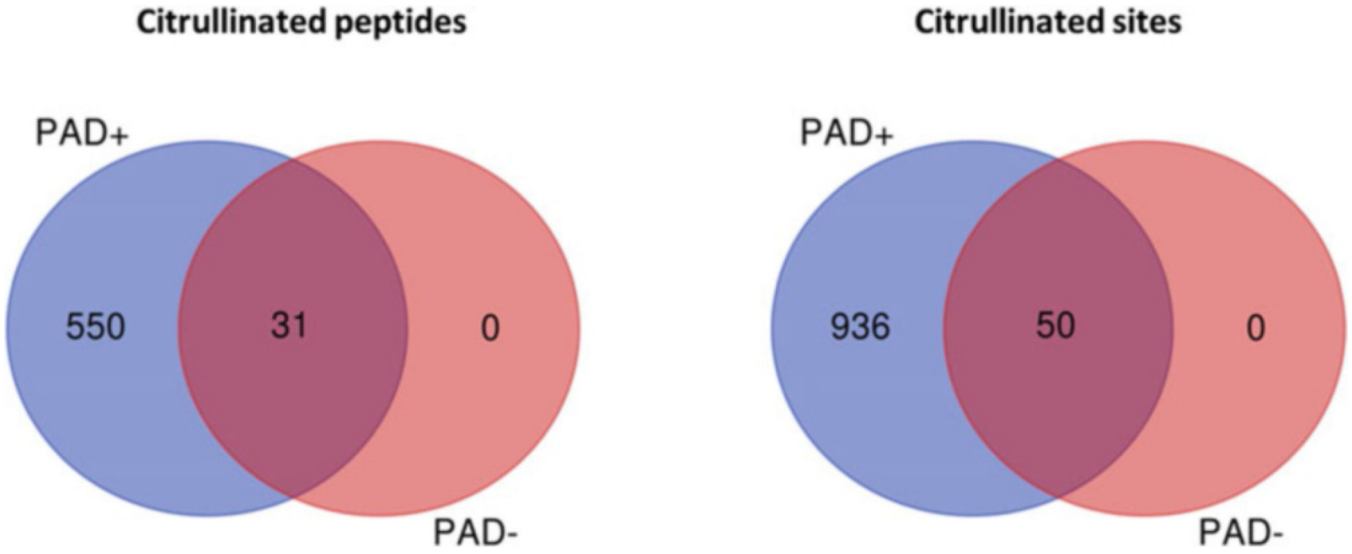

Fig. 2.

Hyper-citrullinated library from THP-1 macrophages: number of citrullinated peptides and sites with and without PAD enrichment (PAD+ versus PAD−)

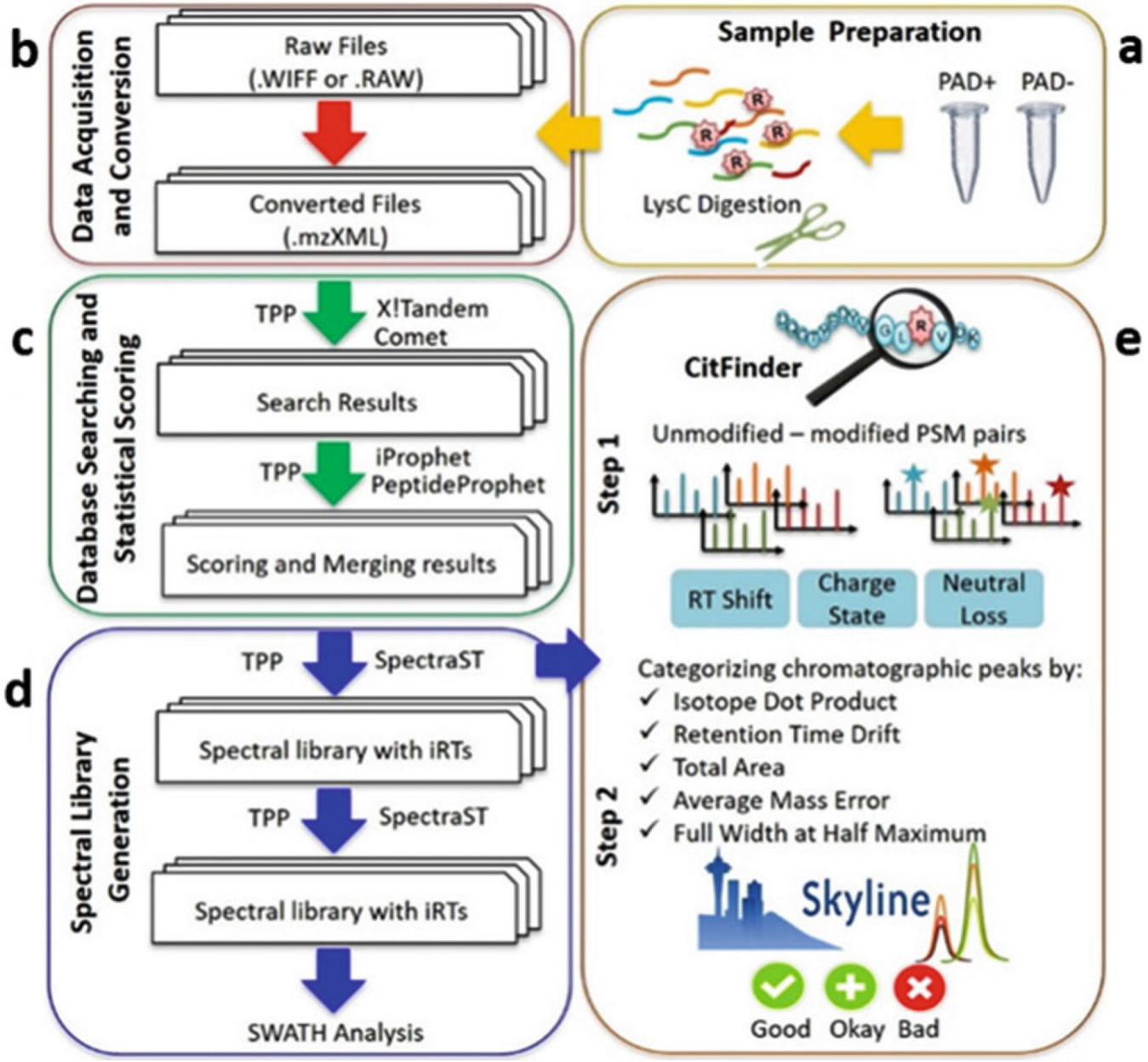

Fig. 3.

Workflow for identification and validation of citrullinated sites: (in anti-clockwise direction, starting from the top right panel) The workflow begins with (**a**) sample preparation followed by (**b**) data acquisition and conversion from raw format to open-source format. (**c**) The fragment ion spectra are searched against a protein sequence database for the identification of PSMs and scored. (**d**) High-scoring PSMs are then converted to spectral library, which is then used by (**e**) CitFinder for the identification and validation of citrullinated peptides. Further, after generation of consensus library from the spectral library, analysis using available SWATH tools can be done

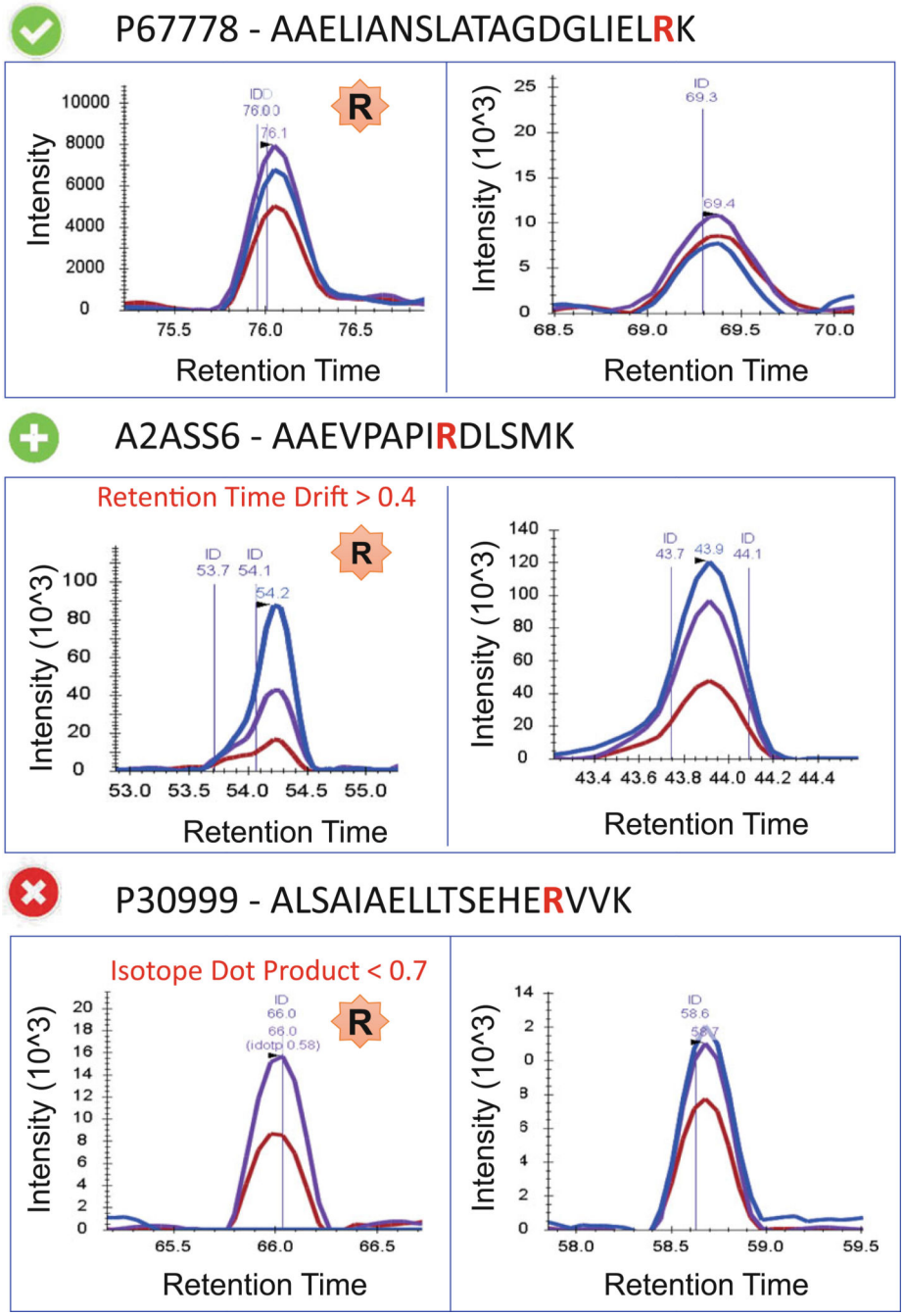

Fig. 4.

Schematic depiction of categorization of modified–unmodified peptide pairs using Skyline: Chromatographic peaks are characterized on the basis of factors such as Isotope Dot Product, Retention Time Drift, Total Area, Total Area/FWHM, and Average Mass Error PPM to be defined as (**a**) “Good,” (**b**) “Okay,” and (**c**) “Bad”

Table 1

The example results of hyper-citrullinated library from THP-1 macrophages: selected results obtained from CitFinder

UniProtKB ID	Protein name	Modified peptide	Unmodified peptide	Citrullinated site	Retention time shift	Charge shift	Total Neutral loss ions
P35579	Myosin-9	QLAAENR[157]LTE METLQSQLMAEK	QLAAENRLTEMETL QSQLMAEK	R867	6.82	Loss, Match	18
P13073	Cytochrome c oxidase subunit 4 isoform 1, mitochondrial	SEDFSLPAYMDR[157]R[157]D HPLPEVAHVK	SEDFSLPAYMD RRDHPLPEVAHVK	R41, R42	5.17	Loss, Match	22
P51991	Heterogeneous nuclear ribonucleoprotein A3	GGSFGGR[157]SSGSPYGG GYGSGGGSGGYGSR[157]R [157]F	GGSFGGRSSGSP YGGGYGSGGGSGG YGSRRF	R354, R376, R377	8.9	Loss	53
Q15149	Plectin	AQVEQELTTLR[157]LQLEET DHQK	AQVEQELTTLRL QLEETDHQK	R2329	5.43	Match	10
P34897	Serine hydroxymethyltransferase, mitochondrial	GLDGAR[157]AER[157]VLELV SITANK	GLDGARAERVLEL VSITANK	R395,R398	11.56	Loss	10

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