

# SIESTA as a universal unbiased proteomics approach for identification and prioritization of enzyme substrates

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**Method Article**

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# Abstract

This protocol describes the proteomics technique called System-wide Identification and prioritization of Enzyme Substrates by Thermal Analysis or SIESTA <sup>1,2</sup>. SIESTA can be used for universal discovery of enzyme substrates that shift in thermal stability or solubility upon post-translational modification (PTM). Experimental design, proteomics sample preparation and data analysis are the key stages of this protocol. Data analysis can be performed using our SIESTA package hosted on GitHub <sup>3</sup>. When performed with classical thermal proteome profiling (TPP), the protocol will take 5 days for sample preparation and 14 days of sample analysis by mass spectrometry (the current protocol). If our high-throughput version of TPP called Proteome Integral Solubility Alteration assay (PISA) <sup>4</sup> is used instead, the sample analysis time by mass spectrometry is reduced to 1-2 days for the same number of conditions.

## Introduction

The identification let alone prioritization of enzyme substrates is challenging. Current approaches for identifying specific substrates are specific to an enzyme or a class of enzymes and are often labor-intensive and not straightforward. Furthermore, modifying enzymes or co-substrates for capturing substrates can alter the biology of the system under study and produce a bias. Thus, unbiased, general and swift proteome-wide techniques avoiding enzyme or cosubstrate modification are needed.

Here, through an inventive experimental development, we designed a new technique called System-wide Identification and prioritization of Enzyme Substrates by Thermal Analysis (SIESTA) by applying TPP <sup>5</sup> or MS-CETSA <sup>6</sup> to samples treated in parallel with vehicle, the enzyme, co-substrate or both. Since PTMs can alter the thermal stability of a protein, SIESTA can probe the proteome-wide effects on thermal stability of specific PTMs and identify enzyme substrates. In the associated publication, we show that SIESTA can identify the substrates of Thioredoxin reductase 1, protein kinase B (AKT1) and poly-(ADP-ribose) polymerase-10 systems <sup>2</sup>. Since SIESTA can identify changes in stability resulting from even small modifications such as phosphorylation, we believe that it can be applied to most enzymes.

Experimental design, proteomics sample preparation and data analysis are the key stages of this protocol. In this protocol, we will elaborate on experimental design aspects and give a detailed protocol on SIESTA workflow. Finally, our SIESTA package on GitHub <sup>3</sup> can be used for data analysis. Here, we will use the AKT1 system as an example.

## Reagents

A recombinant enzyme (e.g. AKT1) (Carna BioSciences, cat no. 01-401-20N)

Corresponding enzyme co-substrate (e.g. ATP) (Sigma, cat no. GE27-2056-01)

Trypsin-EDTA (for cell detachment)

Phosphate buffered saline (PBS)

HEPES (Sigma, cat no. H3375-100G)

Protease inhibitor cocktail (Roche, cat no. 11697498001)

Phosphatase inhibitors (PhosSTOP) (Sigma, cat no. 4906837001)

Liquid nitrogen

Pierce BCA assay kit (Thermo)

Dithiothreitol (DTT)

Iodoacetamide (IAA)

Methanol

Chloroform

EPPS (Sigma, cat no. E9502-100G)

Lysyl Endopeptidase (LysC) (Promega, cat no. VA1170)

Sequencing-grade trypsin (Promega, cat no. V5111)

Acetonitrile

TMT10plex reagents (Thermo, cat no. 90110)

50% Hydroxylamine (Thermo, cat no. 90115)

Trifluoroacetic acid (TFA)

Formic acid

## **Equipment**

Bench-top centrifuge (Thermo, Heraeus Pico 17)

Ultracentrifuge (Beckman, Optima XPN-80)

ThermoMixer compact (Eppendorf)

SimpliAmp Thermocycler (Thermo)

Sep-Pak cartridges (Waters, cat no. 41115712)

DNA 120 SpeedVac™ Concentrator (Thermo)

XBrigde BEH C18 2.1x150 mm column (Waters, cat no. 186003023)

Dionex Ultimate 3000 LC system (Thermo)

EASY-Spray column (75 µm internal diameter, packed with PepMap C18, 2 µm beads, 100 Å pore size)  
(Thermo)

EASY-nLC 1000 (Thermo)

Dionex UltiMate 3000 UHPLC system (Thermo)

Q Exactive HF mass spectrometer (Thermo)

## Procedure

### Experiment design considerations

SIESTA experiments are based on parallel monitoring of protein stability changes in cell lysate aliquots treated with vehicle, enzyme, its co-substrate, or a combination of the enzyme with co-substrate. Within this design, vehicle-, enzyme-, and co-substrate-treated samples serve as controls. Proteins which demonstrate a specific shift in stability in samples treated with a combination of enzyme and co-substrate are considered the putative substrates of the enzyme under study. If the enzyme has more than one co-substrate, the additional cosubstrate(s) can be used as additional control(s). The concentration of enzyme and its co-substrate should be in the physiological range. SIESTA can be performed using either TPP or PISA workflows. Below is the protocol based on TPP. A schematic workflow can be found in Figure 1 of the associated publications <sup>1,2</sup>.

### Sample preparation

1. Culture cells in multiple 175 cm<sup>2</sup> flasks (Sarstedt), detach with trypsinization and wash twice with PBS.

2. Resuspend in 12 mL 50 mM HEPES pH 7.5, with complete protease inhibitor cocktail and phosphatase inhibitor tablets.
3. Lyse the resuspended cells by five freeze-thaw cycles in multiple 1.5 mL eppendorfs using liquid nitrogen.
4. Centrifuge the cell lysate at 21,000 g for 10 min at 4°C and collect the soluble fraction.
5. Measure protein concentration in the lysate using BCA assay.
6. Distribute the lysate equally into 8 aliquots (1.1-1.2 mL each).
7. Treat each pair of aliquots with vehicle, 500 µM ATP, 500 nM AKT1 or with AKT1+ATP and incubate on a thermomixer at 37°C for 30 min.
8. After incubation, aliquot each replicate into 10 PCR microtubes (100 µL each) and incubate each for 3 min in the thermocycler at the corresponding temperature points of 37, 41, 44, 47, 50, 53, 56, 59, 63, and 67°C.
9. Let the samples cool down for 3 min at room temperature and afterwards keep on ice.
10. Transfer the samples into polycarbonate thickwall tubes and centrifuge at 100,000 g and 4°C for 20 min.
11. After ultracentrifugation, collect the soluble protein fractions and transfer them to new Eppendorf tubes.
12. Measure protein concentration in the samples treated at lowest temperature points (37 and 41°C) using BCA Assay.
13. Determine the volume containing 50 µg of protein in the samples corresponding to 37 and 41°C.
14. Transfer the same volume (calculated above) from each sample to new Eppendorf tubes.
15. Add urea to the final concentration of 4 M.
16. Add DTT to a final concentration of 10 mM and incubate for 1 h at room temperature.
17. Add IAA to a final concentration of 50 mM and incubate for 1 h at room temperature in the dark.
18. Quench the reaction by adding an additional 10 mM of DTT.
19. Add 4 volumes of methanol to the samples and vortex.
20. Add 1 volume of chloroform to the samples and vortex (from this step until step 27, work should be done under a fume hood because of methanol and chloroform).

21. Add 3 volumes of deionized water to the samples. At this stage, a cloudy solution must form.
22. Centrifuge the samples at 21,000 g for 10 min. A pellet must form at the interphase.
23. Discard the aqueous layer above the pellet as much as possible.
24. Add 1 volume of methanol, disrupt the pellet by pipetting and wash the pellet by centrifugation at 21,000 g for 10 min.
25. Discard the supernatant.
26. Let the pellets dry (but do not over-dry the pellets).
27. Dissolve the semi-dry protein pellet in 20 mM EPPS (pH=8.5) with 8 M urea.
28. Dilute urea to 4 M.
29. Dissolve LysC in 20 mM EPPS, add 1:100 w/w enzyme to protein (e.g. 0.5 µg enzyme to 50 µg protein) and incubate the samples at room temperature overnight.
30. Dilute the urea to 1 M.
31. Dissolve sequencing-grade trypsin in 20 mM EPPS and add 1:100 w/w enzyme to protein (e.g. 0.5 µg enzyme to 50 µg protein) and incubate the samples at room temperature for 6 h.
32. Add acetonitrile to a final concentration of 20%.
33. Dissolve TMT10 reagents and add 4x by weight to the samples (200 µg TMT label for 50 µg of protein; each TMT reagent will be added to corresponding samples heated to different temperature points, e.g. TMT126 to 37°C, TMT127N to 41°C and so on).
34. Incubate the samples for 2 h at room temperature.
35. Quench the reaction by addition of 0.5% hydroxylamine for 15 min at room temperature.
36. Pool the labeled samples from each replicate.
37. Dry at least 30% of the samples to reduce the acetonitrile content to <5%.
38. Acidify the samples to pH 1-3 using TFA.
39. Clean the samples using Sep-Pak cartridges, according to the manufacturer protocol.
40. Dry the samples using DNA 120 SpeedVac concentrator.
41. Depending on availability of equipment and the proteome coverage required, the samples can then be fractionated by:

42. Either dissolve each replicate in 0.1% TFA and fractionate into 8 fractions using Pierce High pH Reversed-Phase Peptide Fractionation Kit.

Or: Resuspend the samples in 20 mM ammonium hydroxide and fractionate into 96 fractions on an XBridge BEH C18 2.1x150 mm column, using a Dionex Ultimate 3000 LC system. The samples can then be concatenated into as many fractions as desired (for example, for concatenation into 24 fractions as in our paper, fractions 1, 25, 49 and 73 can be combined, as well as fractions 2, 26, 50 and 74, and so on).

43. Dry the samples in a concentrator.

44. Store peptide pellets at -80°C.

### **Liquid Chromatography and Mass Spectrometry**

45. Dissolve the samples in buffer A (0.1% formic acid and 2% acetonitrile in water) to an estimated 0.2 µg/µl concentration.

46. Prepare liquid chromatograph (Dionex UltiMate 3000 UHPLC system). Prepare buffer A (0.1% formic acid and 2% acetonitrile in water) and buffer B (0.1% formic acid, 98% acetonitrile in water).

47. Load 5 µL of each sample. Perform reverse-phase separation using an Easy-Spray HPLC column (75 µm internal diameter, packed with PepMap C18, 2 µm beads, 100 Å pore size).

48. Connect the EASY-Spray column to a nanoflow Dionex UltiMate 3000 UHPLC system (Thermo) and elute in an organic solvent gradient increasing from 4% to 26% (B: 98% acetonitrile, 0.1% formic acid, 2% water) at a flow rate of 300 nL min<sup>-1</sup> over 95 min.

49. For MS acquisition on Q Exactive HF, program the following setting in the method: Record the precursor ion spectra at the m/z range of 375-1500 with 120,000 m/z resolution, automatic gain control target of 3x10<sup>6</sup> ions and maximum injection time of 100 ms. Record fragment ions in MS/MS with 60,000 m/z resolution, automatic gain control target of 2 x10<sup>5</sup> ions and maximum injection time of 120 ms.

### **Data processing**

50. Process the mass spectra raw files using MaxQuant <sup>7</sup> (e.g. v. 1.5.6.5 or 1.6.2.3). Use the UniProt complete human proteome database for protein identification. Use Trypsin/P as enzyme specificity and

do not allow for more than 2 missed cleavages. Select TMT10-plex in MS2 for peptide quantification. Set cysteine carbamidomethylation as a fixed modification, and methionine oxidation as a variable modification. Include phosphorylation on serine and threonine as variable modifications and use the same settings in quantification (the last setting is important if a kinase such as AKT1 is used as an enzyme). Use a 1% false discovery rate as a filter at both protein and peptide levels. Use the “Match Between Runs” option with default settings.

51. Analyze the “ProteinGroups.txt” file resulting from the above search using our SIESTA package hosted on GitHub <sup>3</sup>.

## Troubleshooting

There are a few steps that might need troubleshooting:

### Step 5

Problem: Little protein amount after BCA.

Possible reason: Low efficiency of extraction or low number of starting cells.

Solution: Culture and extract more cells or perform freeze-thaw in smaller Eppendorf to enhance the extraction efficiency.

### Steps 22 and 25

Problem: No pellet is seen.

Possible reason: The pellet is too small.

Solution: This may happen if the sample size is small. Collect 90% of the soluble fraction in step 11.

### Step 25

Problem: Pellet is floating.

Possible reason: Pellet is not disrupted before final centrifugation.

Solution: Fully disrupt the pellet before the final wash.

## Step 50

Problem: Very few peptides are detected.

Possible reason: Failed digestion, wrong trypsin or LysC concentration.

Solution: Repeat the experiment using reagents prepared from stock anew.

## Time Taken

The protocol will take 5 days for sample preparation and 14 days of sample analysis by mass spectrometry (when analyzing 24 fractions, including the blank samples for washing the column between the samples). If our new approach Proteome Integral Solubility Alteration assay (PISA) <sup>4</sup> is used instead, the sample preparation time will reduce by 1 day and analysis time by mass spectrometry is reduced to 1-2 days for the same number of samples. A detailed PISA protocol can be found in the above citation.

## Anticipated Results

This protocol will yield information on the abundance of 5,000-8,000 proteins, depending on the cell line, number of analyzed fractions and the mass spectrometer (and all the settings). The results would identify the co-substrate (here ATP) binding proteins, protein-protein interactions (for AKT1) and putative protein substrates for AKT1. Exemplary results can be seen in our paper in BioRxiv <sup>1</sup> as well as the associated publication <sup>2</sup>.

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