STANDARD OPERATING PROCEDURE

Title: Trypsin digestion of serum and enrichment of N-glycopeptides using an automated liquid handler

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Author: Hui Zhang Lab

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Purpose

The purpose of this document is to describe the enzymatic digestion of serum and the subsequent solid phase enrichment of N-glycopeptides using an automated liquid handler to prepare samples that are compatible with mass spectrometry analysis.

Scope

This procedure is used to reduce, alkylate, proteolyze and enrich N-linked glycopeptides from serum.

Responsibilities

It is the responsibility of person(s) performing this procedure to be familiar with laboratory safety procedures. The interpretation of results must be done by a person trained in the procedure and familiar with such interpretation.

Equipment

- Versette liquid handling system (Thermo Fisher Scientific)
- Vacuum centrifuge

Materials

- Disposable automation research pipette tips (Thermo Fisher Scientific)
- 96-well plate
- Affi-gel hydrazide gel (Bio-Rad)
- Frits (2 mm diameter, 1 mm thick, 15 – 45 µm pores) (POREX)
- 7k MWCO Zeba spin desalting columns (Thermo Fisher Scientific)
- Human Serum (Sigma-Aldrich)

Reagents

- Sodium periodate (Bio-Rad)
- Urea (Thermo Fisher Scientific)
- Tris(2-carboxyethyl)phosphine (Thermo Fisher Scientific)
- Iodoacetamide (Sigma-Aldrich)
- Sequencing-grade trypsin (Promega)
- PNGase F (New England Biolabs)
- Sodium acetate (Sigma-Aldrich)
- Sodium chloride (Sigma-Aldrich)
- Aniline (Sigma-Aldrich)
- Ammonium bicarbonate (NH_4HCO_3) (Sigma-Aldrich)
- Acetonitrile (Fisher Scientific)
- Formic acid (Fisher Scientific)
- Water (Fisher Scientific)
- Trifluoroacetic acid (Thermo Pierce Scientific)

Solutions

- Coupling buffer: 500 mM sodium acetate, 0.3 mM sodium chloride, pH 5
- Oxidation buffer: 15 mM sodium periodate
- 100 mM aniline
- Urea buffer: 8 M urea in 0.4 M NH_4HCO_3
- 10 mM TCEP
- 12 mM iodoacetamide
- 1.5 M sodium chloride
- 80% acetonitrile
- 50% acetonitrile
- 25 mM NH_4HCO_3
- 100 mM NH_4HCO_3

Procedure

1. Prepare hydrazide pipette tips
    a. Push one frit into a disposable automation research tip (DART)
    b. Load 200 µL hydrazide resin (50% slurry) into each pipette tip
    c. Force any residual liquid out of the tip
    d. Push one 5 mm diameter frit into the tip to secure the hydrazide resin between the two frits
    e. Wash each tip 5x with 200 µL deionized water

f. Condition each tip 5x with coupling buffer
g. Note: Discard tips with slow flow rates due to high resistance

2. Couple glycoproteins to hydrazide resin
    a. Note: The loading capacity of hydrazide beads is ~ 40 µL serum/200 µL hydrazide beads (50% slurry), and the average glycopeptide yield per 40 µL of serum is 20 µg.
    b. Dilute serum 1:1 with oxidation buffer
    c. Oxidize with oxidation buffer containing 100 mM aniline for 1 h at room temperature in the dark
    d. Buffer exchange with coupling buffer using Zeba spin desalting column
    e. Slowly aspirate serum sample into hydrazide tips and dispense into 96-well plate for 30 min using Versette

3. Denature, reduce, alkylate and proteolyze glycoproteins
    a. Wash serum coupled to the hydrazide tips via their oxidized glycans with 3 mL of urea buffer
    b. Reduce disulfide bonds with 10 mM TCEP for 30 min
    c. Alkylate with 12 mM iodoacetamide for 15 min in the dark at room temperature
    d. Wash with 3 mL of urea buffer
    e. Digest with trypsin (1:120) in 100 mM NH_4HCO_3 for 1 h

4. Remove residual non-glycopeptides released by trypsin digestion
    a. Wash hydrazide tips (containing conjugated glycopeptides) with 6 mL of 1.5 M sodium chloride
    b. Wash hydrazide tips with 6 mL of 80% ACN
    c. Wash hydrazide tips with 6 mL of deionized water
    d. Wash hydrazide tips with 6 mL of 25 mM NH_4HCO_3

5. Release N-linked glycopeptides
    a. Release glycopeptides with 1500 U PNGase F in 25 mM NH_4HCO_3 for 1 h at room temperature
    b. Wash tips 3x with 50% ACN
    c. Combine eluents and dry in a vacuum centrifuge

6. Reconstitute samples
    a. Re-constitute in 1% TFA
    b. Measure peptide concentration using NanoDrop
    c. Dilute to working concentration with 2% ACN/0.2% formic acid

Referenced Documents

Chen J, Shah P, Zhang H. Solid phase extraction of N-linked glycopeptides using hydrazide tip. *Analytical Chemistry*. 2013 (85):10670-10674. PMID: 24079330