|  |
| --- |
| STANDARD OPERATING PROCEDURE |
|

|  |
| --- |
| **Title: Preparation of clinical serum samples as background matrix for targeted mass spectrometry analysis** |
| **Version #: 1.1** | **Author: Hui Zhang Laboratory – Johns Hopkins University** |
| **Date: 04/05/2017** |  |

 |

**Purpose**

The purpose of this document is to describe the enzymatic digestion method of serum for the preparation of clinical serum samples that will be used as the background matrix for targeted mass spectrometry-based analytical methods.

**Scope**

This document describes the detailed procedures for reduction, alkylation, proteolysis, and peptide desalting.

**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**

* Temperature-controlled shaking incubator
* Speed-Vac
* Lab rotator
* NanoDrop spectrophotometer

**Materials**

* 1 g 6 cc C18 SPE cartridges (Waters)

**Reagents**

* Deionized water
* Urea – Ultra Pure (Thermo Fisher Scientific)
* TCEP (Thermo Fisher Scientific)
* Iodoacetamide (Sigma-Aldrich)
* Optima LC/MS-grade water (Thermo Fisher Scientific)
* Bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific)
* Sequencing-grade modified trypsin (Promega)
* Trifluoroacetic acid (Sigma-Aldrich)
* Ammonium bicarbonate (Sigma-Aldrich)
* Formic acid (Sigma-Aldrich)
* LC/MS-grade water (Thermo Fisher Scientific)
* LC/MS-grade acetonitrile (Thermo Fisher Scientific)

**Solutions**

* Lysis buffer: 8 M urea, 1 M NH4HCO3, pH 8.0
* 500 mM TCEP
* 300 mM Iodoacetamide
* 0.1 % TFA
* 50 % ACN/0.1 % TFA
* 60 % ACN/0.1 % TFA
* Trypsin (0.5 µg/µL)

**Procedure**

1. **Reduction with TCEP**
	1. Dilute serum samples (~20 mg) 1:10 with urea buffer (8M urea, 1M NH4HCO3, pH 8.0) before starting BCA assay.
	2. Measure the protein concentration using the BCA protein assay kit.
	3. Add TCEP to samples to reach 10 mM in solution (using 500 mM TCEP stock).
	4. Incubate the sample at 37 °C for 1 h.
2. **Alkylation with Iodoacetamide (IAA)**
	1. Make 300 mM stock solution of IAA. (Prepare fresh).
	2. Add 1/20 of the final volume of 300 mM IAA to samples (Final concentration ~ 15 mM IAA)
	3. Wrap completely with foil (IAM is light sensitive), and incubate in a lab rotator for 30 min at room temperature.
3. **Proteolysis (Trypsin digestion)**
	1. Dilute sample 6-fold by adding H2O (1.3 M final urea concentration and 166 mM NH4HCO3).
	2. Add trypsin (1 : 40 = trypsin : protein (w/w))
	3. Incubate 12 h at 37 °C with gentle shaking.
4. **Peptide de-salting**
	1. Adjust the pH of digested samples to < 3 with 50 % TFA. (The final concentration of TFA is ~1.3 - 1.5 %).
	2. Centrifuge the digested samples under 3000 *g* for 5 mins to pellet the non-digested material. Preserve the supernatant for de-salting.
	3. Condition the C18 column with 6 mL 80 % ACN/0.1 % TFA 3 times.
	4. Equilibrate the C18 column with 6 mL of 0.1 % TFA 2 times.
	5. Load the sample onto the C18 column.
	6. Collect the flow through, and reload the flow through.
	7. Wash the column with 6 mL of 0.1 % TFA 3 times
	8. Elute by 1 mL of 60 % ACN/0.1 % TFA 4 times.
	9. Determine the concentration by nanodrop.
	10. Dry in Speed-Vac

**Referenced Documents**

1. Sun S, Zhou JY, Yang W, Zhang H. Inhibition of protein carbamylation in urea solution using ammonium-containing buffers. Anal Biochem. 2014 Feb 1;446:76-81. doi: 10.1016/j.ab.2013.10.024. Epub 2013 Oct 23. PubMed PMID: 24161613; PubMed Central PMCID: PMC4072244.