STANDARD OPERATING PROCEDURE

Title: Validation samples preparation (Assessment of repeatability)

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Purpose

The purpose of this document is to describe the characterization of a set of assays according to its repeatability of measurement over 5 days. This is to estimate the performance of the assay measured in a complex sample over multiple days.

Scope

This procedure covers overall preparation and running of samples for generating the validation samples with regards to CPTAC Assay Characterization Guidance experiment #2.

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

- Waters positive pressure displacement 96 manifold.

Materials

- HPLC water: Fisher Cat# W6-4
- Formic Acid: EDM Cat# 11670-1
- Acetonitrile: Fisher Cat# A955-4

Reagents

Standards:

- Heavy stable isotope-labeled standards (SIS) were synthesized with a fully atom labeled 13C and 15N isotope incorporated at the C-terminal lysine (K) or arginine (R) position of each (tryptic) peptide, resulting in a mass shift of +8 or +10 Da, respectively. Pure standards were purified to >95% purity by HPLC and quantified by amino acid analysis. Aliquots were stored in 3% acetonitrile/0.1% formic acid at -80°C until use. Crude peptides were synthesized on a 1.0 µmol scale, purified by solid phase extraction to remove all non-peptide contaminants (average purity ~75%), and the correct molecular mass verified by MS. Aliquots were shipped lyophilized and resuspended in 1 mL 3% MeCN/0.1% FA or were shipped in 0.4 mL 50% MeCN/0.1% TFA. Aliquots were stored at −80°C until use.
- The stock heavy SIS mix is at 100 nM. Crude concentrations were estimated assuming a 10% synthetic yield.

Matrix:

- An equal mix (by total protein mass) of MCF10A + 10Gy IR, MCF10A + 0.5mM MMS, and PBMCs + 10Gy IR was used as a background matrix. Lysates were prepared as described in SOP P-01 (Cell line lysis). The pooled lysate was aliquotted and independent digestions were performed for each replicate of repeatability validation to make complete process replicates for each sample. Following digestion, heavy synthetic peptide was added to the samples at three concentrations (low, med, high) and the samples were desalted and the phosphopeptides enriched by IMAC as described in SOP P-03 (Phosphopeptide enrichment). Measurements of heavy:light (endogenous) ratio were used as validation of repeatability at the concentrations described below. Digests for different days are performed >= 24 hours apart.

Procedure

Heavy SIS spike levels and preparation of samples

1. Heavy peptides were spiked into 200µg of the digested lysate at three concentrations: low, medium and high.
    a. High – 100 nM
    b. Med – 5 nM
    c. Low – 0.25 nM
2. The stock heavy SIS mix at 100nM is serially diluted with 0.1% formic acid / 3% acetonitrile to create the three concentrations above.
3. 10 µL of each concentration point of heavy SIS mix is added to independently digested aliquots (see SOP D-04 for digestion protocol) of the background matrix to create five replicates of low, med, and high concentrations of heavy peptide for each day. Each aliquot of background matrix contains 200ug of total protein.
4. The digested matrix plus heavy peptides are desalted (see step B).

Desalting Samples Offline by Positive Pressure

5. Set the system pressure to 80psi.
6. Condition desalting plate with 3 x 400µL of 0.1% formic acid in 80% ACN at 12psi.

7. Equilibrate desalting plate with 4 x 400µL of 0.1% formic acid in 100% water at 12psi.
8. Add sample to desalting plate at 12psi.
9. Wash with 4 x 400µL of 0.1% formic acid in 100% water at 6psi.
10. Elute peptides with 3 x 400µL 0.1% formic acid in 80% acetonitrile at 3psi into a deep well plate.
11. Freeze eluates on dry ice or at -80°C for approximately 1 hour.
12. Lyophilize samples overnight to dryness.
13. Samples can be stored lyophilized at -80°C until ready for IMAC enrichment as described in SOP P-03 (Phosphopeptide enrichment).

Referenced Documents

SOP D-04 Trypsin digestion of cell lysate.pdf
SOP P-01 Cell line lysis.pdf
SOP P-03 Phosphopeptide enrichment.pdf