

STANDARD OPERATING PROCEDURE

Title: Response curve

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Purpose

The purpose of this document is to describe the characterization of a set of assays by response curve.

Scope

This procedure covers overall preparation and running of samples for generating the response curve.

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 ^{13}C and ^{15}N 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 200 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 in an automated fashion as described in SOP D-01 (Trypsin digestion of cell lysate) for each replicate of concentration points to make complete process replicates for each response curve. Following digestion, heavy synthetic peptide was added to the samples at one of eight concentrations, 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 to characterize the assays at the concentrations described below. Digests for different days are performed \geq 24 hours apart.

Procedure

Heavy SIS spike levels and preparation of samples

1. Heavy peptides were spiked into 200μg of the digested lysate at 8 points of varying concentrations of analyte and 2 blanks. All prepared in triplicate.
2. The stock heavy SIS mix at 200 nM is serially diluted with 0.1% formic acid / 3% with the first three points using 10-fold serial dilutions and the rest 2.5-fold, over the range 200-0.020 nM.
3. 10 μL of each concentration point of heavy SIS mix is added to a series of independently digested aliquots (see SOP D-04 for digestion protocol) of the background matrix to create the eight-point response curve and two blanks 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 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