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| STANDARD OPERATING PROCEDURE |
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| **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
* microcentrifuge

# Materials

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

# Reagents

**Standards:**

* Custom synthetic peptide standards were obtained from New England Peptide, 21st Century Biochemicals, or Thermo Biopolymers.
* Heavystable isotope-labeled standards (SIS) and matched light versions were synthesized and purified to >95% purity by HPLC. Heavy peptides incorporated a fully atom labeled 13C and 15N isotope 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. Peptides were quantified by amino acid analysis and aliquots were stored in 30% acetonitrile/0.1% formic acid at -80°C until use.
* The stock heavy SIS mix is at 100 nM
* The stock light peptide mix is at 50nM

**Matrix:**

* A background matrix consisting of an equal mix (by protein mass) of 30 breast cancer-related cell lines was prepared. Lysates were prepared as described in SOP P-1 (Cell line lysis). Digestion was performed in an automated fashion as described in SOP D-02 (Trypsin digestion in cell lysate). The pooled digest was used for generating the background matrix in the response curves.

# Procedure

**A. Preparation of samples**

1. The following is designed to create 8 points of varying concentrations of analyte, 1 blank, and 1 double blank. All prepared in triplicate.
2. The stock heavy SIS mix at 100nM is serially diluted with buffer to create a series of concentrations. Starting with 1 mL of heavy SIS mix, take 330uL and mix into 660uL 0.1% formic acid / 3% acetonitrile. Continue this operation with the new sample for a total of 8 concentration points. The same pipettor tip is used for the serial dilution.
3. 200 uL of each concentration point of heavy SIS mix is added to a series of each samples of the digested background matrix (two are blanks), each aliquot of matrix containing 100 ug of total protein digest.
4. 10 uL of the stock light peptide mix at 50nM is added to nine series of the samples of background matrix for a concentration of 5fmol/ug.
5. The samples containing background matrix, heavy SIS, and light synthetic peptide are desalted according to below.

**B. Desalting Samples Offline by Positive Pressure**

1. Set the system pressure to 80psi.
2. Condition desalting plate with 3 x 400μL of 0.1% formic acid in 80% ACN at 12psi.
3. Equilibrate desalting plate with 4 x 400μL of 0.1% formic acid in 100% water at 12psi.
4. Add sample to desalting plate at 12psi.
5. Wash with 4 x 400μL of 0.1% formic acid in 100% water at 6psi.
6. Elute peptides with3 x 400μL 0.1% formic acid in 80% acetonitrile at 3psi into a deep well plate.
7. Freeze eluates on dry ice or at -80°C for approximately 1 hour.
8. Lyophilize samples overnight to dryness.
9. Samples can be stored lyophilized at -80°C until ready for SRM analysis.

**C. Reconstituting Samples (To be performed just prior to executing LC-SRM)**

1. Reconstitute dried and desalted digests with 100μL of 3% acetonitrile, 0.1% formic acid to each sample to achieve 1μg/μL digest solution for the cell lysates.
2. Vortex sample, spin down, and transfer ~95μL to an autosampler vial.
3. LCMS analysis is performed according to SOP LC-01 and SOP MS-01.

**D. Run Order**

1. Samples are run in increasing order of concentration, one pass per replicate. System suitability standards (SSS) are acquired in between replicates to check instrument performance. A total of 3 replicates is acquired for each concentration.

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| **Sample** |
| **Initial conditions solvent blank (2x for dual column systems)** |
| **SSS replicate (2x for dual column systems)** |
| **Curve double blank** |
| **Curve blank** |
| **0.09nM** |
| **0.27nM** |
| **0.82nM** |
| **2.5nM** |
| **7.4nM** |
| **22.2nM** |
| **66.7nM** |
| **200nM** |
| **Wash (2x for dual column systems)** |

# Referenced Documents

SOP D-02 Trypsin digestion in cell lysate.pdf

SOP LC-01 Liquid Chromatography Ultra nanoflex dual col.pdf

SOP MS-01 peptide MRM on 5500 QTRAP.pdf

SOP P-01 Cell Line Lysis.pdf