Application Note

Global protein phosphorylation analysis with heat stabilized samples

Introduction

The use of bottom-up proteomics, especially with focus on protein phosphorylation analysis, for the molecular classification of tumors and for biomarker discovery is rapidly gaining acceptance in basic and clinical research. However, the removal of a biological sample for analysis induce a cascade of enzymatically driven changes causing the molecular profile of the sample to deviate from the *in vivo* profile. This can lead to change in disease related biomarkers and erroneous conclusions. In order to avoid post-sampling change and preserve a sample quality that enables an analysis of the molecular state closer to *in vivo*, heat induced protein denaturation using the Stabilizor™ system, has been introduced as a means to preserve the sample. In this application note, results from two bottom-up proteomic experiments focused on protein phosphorylations are high-lighted. Both are affinity enrichment studies aimed at showing the difference between using heat stabilized (HS) tissue samples and standard practice (SF) treated tissue samples.

Benefits

- Preserves levels of protein phosphorylation
- Enable detection of unstable biomarkers
- Increasing the accuracy of quantification analysis
- Additive-free method, for all tissue types, fresh or frozen, and biofluids
- Ensures quality and standardization of sample collection

Study 1: Comparative MS analysis of antibody affinity isolated pTyr peptides

A large number of pTyr peptides with higher levels, or only found in HS samples, show dynamic levels of phosphorylations indicating involvment in cell signaling.

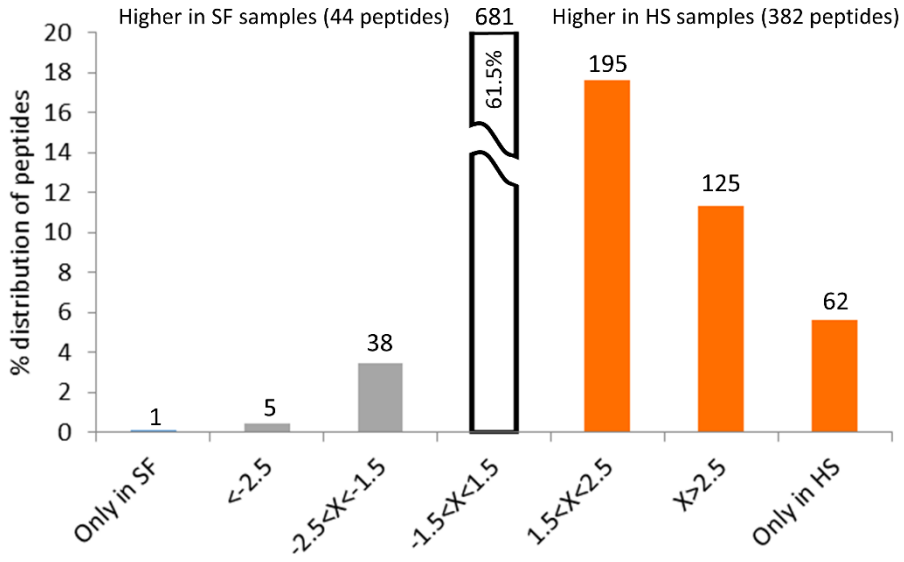

Figure 1. Intensity ratio distribution (X) of pTyr containing tryptic peptides between heat stabilized (HS) and standard (SF) mouse brain samples. Almost 40 % of detected peptides show more than 50 % intensity difference between treatments. 62 pTyr containing peptides was only identified in HS samples. (Number of peptides above bars)

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Study 2: TiO2 enrichment and MS analysis of phosphorylated peptides from human liver

An additional 33% phosphorylated peptides was discovered in human liver samples when using heat inactivation of enzymes. Heat stabilization preserves phosphorylations that would otherwise be lost and enables detection of a substantially higher number of phosphorylated peptides.

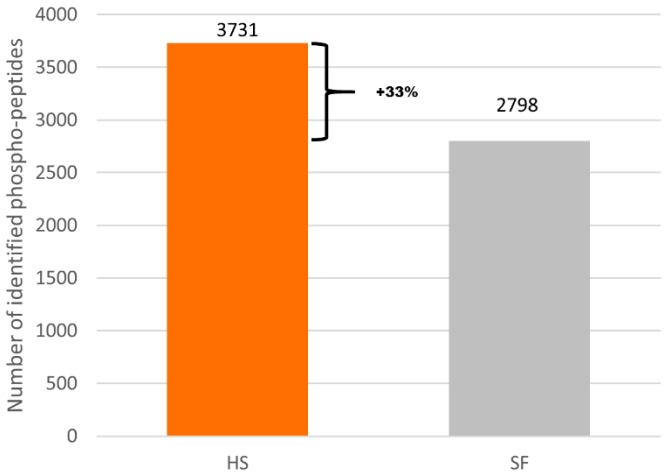

Figure 2. Number of phosphorylated peptides detected from human liver samples either heat stabilized (HS) or rapidly frozen (SF) post sampling (N=5). Prior to sampling the explanted liver had a 30 min room temperature exposure during transfer from the OR to the pathology lab which likely reduce the overall number of detected phosphorylations but emphasize the advantage of using heat inactivation also on samples with long post-sampling time as phosphorylations are continuously lost also during sample work-up.

Materials and methods

Two bottom-up phospho shotgun experiments have been carried out in collaboration with leading CROs in Europe and the USA where the merits of heat induced inactivation of enzymes have been evaluated head to head with best practice routines.

Study 1. pY peptides, mouse brain samples (N=3x2 for each group) were either directly snap frozen in liquid nitrogen or first heat stabilized prior to snap freezing in liquid nitrogen. Tissue samples were homogenized with a Polytron tissue homogenizer for 20 s, sonicated for 20 s at 10 w, cooled on ice for 1 min, 3-times; and clarified by centrifugation at 20,000g for 15 min under denaturing conditions (9 M Urea, 20mM HEPES pH 8.0). The soluble protein extracts were reduced, alkylated and digested with trypsin (1:100) overnight. Peptides were purified by reverse phase C18 column chromatography and lyophilized in 5 mg aliquots. Peptides were resuspended in immunoaffnity purification (IAP) buffer (50 mM MOPS pH 7.2, 10 mM KH2PO4, 50 mM NaCl) and phosphotyrosine target peptides were immunoprecipitated using pY1000 antibody coupled to protein A agarose beads for 2 hrs. Peptides were released by 0.15% TFA 2X and desalted over a C18 StageTip column and dried under vacuum prior to LC-MS analysis .

Study 2. Phosphorylated peptides, three discarded livers from human liver transplants were used having roughly 30 min post-excision time on wet ice. Five core needle biopsies were taken from non-tumor regions and heat stabilized. An additional five core needle biopsies were snap frozen by transferring tissue to Eppendorf tubes that were subsequently snap frozen in a -70ºC isopentane bath. The biopsies were weighed then cryopulverized using a MultiSample BioPulveriser (MSBP, Biospec). To the frozen powder, ice cold lysis buffer (20 µl/mg of tissue) were added (8M urea, 75 mM NaCl, 50 mM Tris-pH 8.2) containing inhibitor cocktails (complete mini and PhosSTOP phosphatase inhibitor cocktails, Roche). Samples were then sonicated using a Sonifier (Branson, W450D) at 15% Amplitude for 3 x 10 s, pulsing on and o, on ice (4°C). 1 mg of total protein was used for each of the ten samples for the SysQuant workflow including trypsin cleavage, 10-plex TMT labelling, TiO2 enrichment of phosphorylated peptidesand analysis on an Orbitrap FusionTM TribridTM Mass Spectrometer (ThermoFisher).

Both studies were conducted by leading CROs using their standard workflows.