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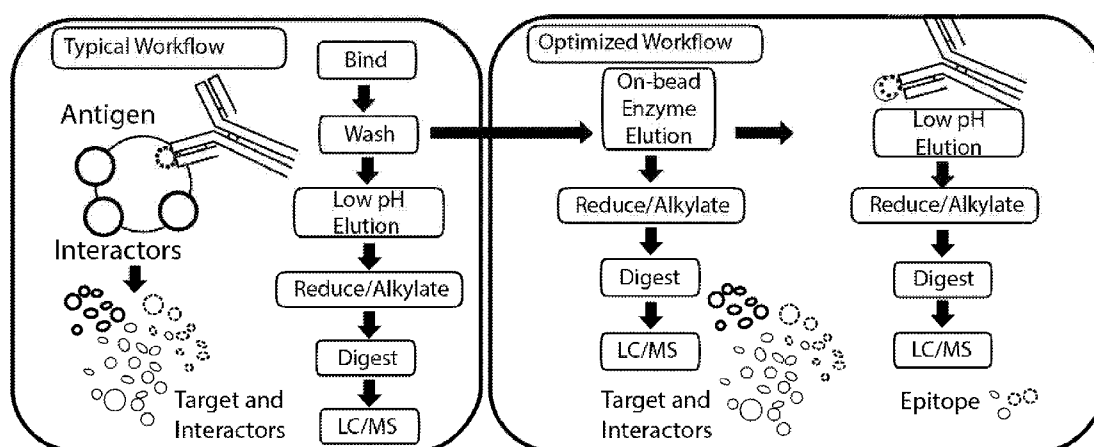


Figure 1

(57) Abstract: This disclosure relates to the field of mass spectrometry analysis. In some embodiments, the disclosure relates to methods for detecting and quantifying proteins by enrichment followed by mass spectrometry analysis.

IMPROVED METHODS FOR IMMUNOAFFINITY ENRICHMENT AND MASS SPECTROMETRY

FIELD OF THE INVENTION

[0001] This disclosure relates to the field of detection and quantification of proteins, by immunoaffinity enrichment and mass spectrometry.

BACKGROUND

[0002] Mass spectrometry (MS) is increasingly becoming the detection methodology of choice for assaying protein abundance and post-translational modifications. Immunoprecipitation (IP) is commonly used upstream of MS as an enrichment tool for low-abundant protein targets. *See*, Gingras et al., *Nat. Rev. Mol. Cell. Biol.*, Aug 2007, 8 (8), 645–54; and Carr, S. A. et al., *Mol. Cell. Proteomics* Mar 2014, 13 (3), 907–17. Additional methods of enriching for the protein of interest upstream of MS may also be used. *See*, e.g., Lin, et al., *J. Proteome Res.*, 2013 Dec. 6; 12(12);5996-6003; Schwertman, et al., *Analytical Biochemistry*, Vol. 440, Issue 2, 15 September 2013, 227-236; and Rafalko, et al., *Anal. Chem.*, 2010, 82(21), 8998-9005.

[0003] The present disclosure provides methods for detecting and quantifying proteins via immunoaffinity enrichment, mass spectrometry (MS), and immunoaffinity enrichment followed by mass spectrometry (IP-MS).

SUMMARY

[0004] In some embodiments, methods are provided for detecting one or more target protein(s) in a biological sample. In some embodiments, methods are provided comprising enriching the target protein(s) from a biological sample by binding the target proteins(s) to a solid support. In embodiments, methods are provided comprising fragmenting enriched target protein(s). In embodiments, methods are provided comprising while bound to the solid support, treating enriched target protein(s) with a first enzymatic digestion, reducing and alkylating the digested target protein(s) concurrently in a single reaction vessel, digesting the reduced, alkylated, and digested target protein(s) in a second enzymatic digestion, wherein optionally the second enzymatic digestion is allowed to proceed for up to 18 hours (for example up to 4 hours). In some embodiments, methods are provided comprising detecting one or more target protein(s) in the sample. In some embodiments, in which enriching the target protein(s) from a biological sample by binding the target protein(s) to a solid support comprises treating the biological

sample with at least one antibody capable of immunoaffinity enriching the target protein(s) from a biological sample. In some embodiments, detecting one or more target protein(s) in the sample comprises assaying the fragmented protein(s) via mass spectrometry to determine the presence or absence of at least one peptide from the target protein(s) and detecting one or more target protein(s). In some embodiments, detecting one or more target protein(s) in the sample comprises ELISA, Western blot, bead-based multianalyte profiling (such as Luminex), fluorescence-based imaging, or chemiluminescent-based imaging.

[0005] In embodiments, methods are provided in which the reduction and alkylation occurs in a single reaction vessel. In embodiments, methods are provided in which the second enzymatic digestion is allowed to proceed for up to 18 hours (for example up to 4 hours). In some embodiments, the peptide from the target protein(s) is less than or equal to 40 amino acids in length.

[0006] In some embodiments, methods are provided in which the first and/or second enzymatic digestion comprises digestion with trypsin, chymotrypsin, AspN, GluC, LysC, LysN, ArgC, proteinase K, pepsin, clostripain, elastase, GluC biocarb, LysC/P, LysN promisc, protein endopeptidase, staph protease or thermolysin. In some embodiments, the first and/or second enzymatic digestion comprises digestion with trypsin. In some embodiments, the first and/or second enzymatic digestion comprises digestion with trypsin and LysC. In some embodiments, trypsin is present in the first enzymatic digestion at an amount of about 0.5 µg to about 2 µg or a concentration of about 0.1 µg/µl to about 0.4µg/µl. In some embodiments, trypsin is present in the first enzymatic digestion at an amount of about 1 µg/µl or a concentration of about 0.2 µg/µl. In some embodiments, the trypsin is present in the second enzymatic digestion at an amount of about 0.2 µg/µl to 0.8 µg/µl or a concentration of about 0.02 µg/µl to about 0.08 µg/µl. In some embodiments, the trypsin is present in the second enzymatic digestion at an amount of about 0.6 µg/µl or a concentration of about 0.06 µg/µl.

[0007] In some embodiments, methods are provided in which the reduction/alkylation step comprises mixing the product of the first enzymatic digestion with a solution comprising TCEP and chloroacetamide. In some embodiments, the TCEP and chloroacetamide are present in a ratio of 1:1, 1:2, 1:3, 1:4, or 1:5. In some embodiments, TCEP is present in a concentration of about 5 to about 10 mM. In some embodiments, chloroacetamide is present in a concentration of about 5 to about 50 mM.

[0008] In some embodiments, the methods further comprise the step of neutralization after the second digestion and prior to mass spectrometry. In some embodiments, the neutralization step comprises adding trifluoroacetic acid (TFA) to the product of the second enzymatic digestion.

[0009] In some embodiments, methods are provided comprising treating the sample with a labelled antibody capable of binding to the target protein to provide a labelled antibody-protein conjugate; and binding the labelled antibody-protein conjugate with a capture agent capable of binding to the labelled antibody to isolate the target protein from the sample. In some embodiments, the label is biotin and the capture agent is streptavidin.

[0010] In some embodiments, the lower limit of detection for the protein(s) is from about 0.04 to about 11.11 fmol.

[0011] In some embodiments, methods are provided further comprising determining the quantity of the target protein. In some embodiments, the quantity of a target protein is determined by adding an internal standard peptide of known amount to the digested protein prior to mass spectrometry, wherein the internal standard peptide has the same amino acid sequence as a target peptide, and is detectably labeled, and determining the quantity of a target peptide by comparison to the internal standard. In some embodiments, the quantity of a target protein is determined by a method comprising comparing an amount of a target peptide in the sample to the amount of the same target peptide in a control sample.

[0012] In some embodiments, methods are provided further comprising quantifying the relative amount of the target protein. In some embodiments, methods are provided further comprising quantifying the absolute amount of the target protein. In some embodiments, the lower limit of quantification is from about 0.04 to about 11.11 fmol.

[0013] In some embodiments, methods are provided further comprising desalting after fragmentation and prior to mass spectrometry. In some embodiments, methods are provided further comprising desalting online using C18 trap column prior to liquid chromatography to mass spectrometry analysis.

[0014] In some embodiments, the mass spectrometry may be selected from targeted mass spectrometry and discovery mass spectrometry. In some embodiments, the targeted mass spectrometry may be selected from multiple reaction monitoring (MRM), selected reaction monitoring (SRM), and parallel reaction monitoring (PRM), or combinations thereof.

[0015] In some embodiments, the biological sample may be selected from isolated cells, plasma, serum, whole blood, CSF, urine, sputum, tissue, and tumorous tissue. In some embodiments, the biological sample is human.

[0016] In some embodiments, methods are provided wherein the peptide from the target protein(s) comprises an epitope for the antibody capable of immunoaffinity enriching the target protein(s).

[0017] In some embodiments, methods including assessing completion of digestion. A complete digestion comprises 90% or higher zero missed cleavages.

[0018] In some embodiments, the method further comprises separating the solid support from digested proteins(s).

BRIEF DESCRIPTION OF THE FIGURES

[0019] FIGURE 1 shows a representative improved workflow for an immunoaffinity enriched mass spectrometry assay to identify target proteins.

[0020] FIGURE 2 shows a comparison of representative workflows for processing immunoaffinity enriched samples.

[0021] FIGURE 3 shows the results of MS sample prep method for low pH/organic IP elutions compared with a urea-based method.

[0022] FIGURE 4 shows the results of various conditions for IP elution using enzyme and sequential reduction/alkylation.

[0023] FIGURE 5 shows IgG levels obtained with enzyme elution compared to IP-MS elution buffer methods.

[0024] FIGURE 6 shows the recovery of target peptides (as percent of control) obtained with different processing methods. A urea method is used as a control.

[0025] FIGURE 7 shows recovery of target peptides obtained with urea vs. enzyme elution methods.

[0026] FIGURE 8 compares recovery of peptides using urea-based method, trypsin elution, and trypsin elution with single pot reduction/alkylation.

[0027] FIGURES 9A-B show results for average top peptide area for different enzyme digestion times. An overnight (O/N) digestion was used as a control and the data are shown as a % of control.

[0028] FIGURES 10A-B show results for average peptide area obtained with different enzyme digestion times and digestion temperatures. An overnight (O/N) digestion was used as a control and the data are shown as a % of control.

[0029] FIGURES 11A-C shows %CV (coefficient of variation) of peptide area obtained from three different experiments in which different enzyme digestion times were compared.

[0030] FIGURES 12A-F show the results of targeted MS analysis of unique peptides for each target across different digestion times presented as % of overnight digestion (control). A) mTOR; B) RAS; C) STAT3; D) RPTOR; E) CTNNB1; F) IQGAP1.

[0031] FIGURES 13A-B show the results of A) peptide intensities and B) % 0 missed cleavage for peptides for different amounts of enzyme(s) in digestions and digestion times. An overnight digestion with 200 ng trypsin was used as a control and the data are shown as a % of control. (T: Trypsin; T+L: Trypsin+LysC)

[0032] FIGURES 14A-F show the results of targeted analysis for unique peptides for each target under different digestion conditions with respect to enzyme, enzyme amount, and time of digestion as compared to an overnight control digestion. A) mTOR; B) RAS; C) STAT3; D) RPTOR; E) CTNNB1; F) IQGAP1.

[0033] FIGURE 15 provides a flowchart outlining an experimental protocol to test conditions for enzymatic elution of immunoprecipitated material from beads with varying amounts of enzyme and time of initial digestion.

[0034] FIGURES 16A-B show the peptides recovered under different conditions of enzyme IP elution from beads. Graphs are plotted as % Control (trypsin elution (E) using 1ug of trypsin for 1hour).

[0035] FIGURES 17A-F show Parallel Reaction Monitoring (PRM) analysis of peptides under different enzyme elution conditions.

[0036] FIGURE 18 shows a flowchart for an experimental protocol to test conditions for the enzymatic elution of immunoprecipitated material from beads. The grade of trypsin, amount of trypsin, and time of elution digestion are varied.

[0037] FIGURES 19A-B show the results of an experiment to optimize the enzymatic elution of immunoprecipitated material from beads. The grade of trypsin, amount of trypsin, and time of elution digestion were varied. (T: trypsin)

[0038] FIGURE 20 shows the %CV from two experiments to test conditions for the enzymatic elution of immunoprecipitated material using trypsin.

[0039] FIGURE 21 summarizes the results from two experiments to test conditions for the enzymatic elution of immunoprecipitated material using 1 µg trypsin.

DETAILED DESCRIPTION

I. Definitions

[0040] This description and exemplary embodiments should not be taken as limiting. For the purposes of this specification and appended claims, unless otherwise indicated, all numbers expressing quantities, percentages, or proportions, and other numerical values used in the specification and claims, are to be understood as being modified in all instances by the term “about,” to the extent they are not already so modified. Accordingly, unless indicated to the contrary, the numerical parameters set forth in the following specification and attached claims are approximations that may vary depending upon the desired properties sought to be obtained. At the very least, and not as an attempt to limit the application of the doctrine of equivalents to the scope of the claims, each numerical parameter should at least be construed in light of the number of reported significant digits and by applying ordinary rounding techniques.

[0041] It is noted that, as used in this specification and the appended claims, the singular forms “a,” “an,” and “the,” and any singular use of any word, include plural referents unless expressly and unequivocally limited to one referent. As used herein, the term “include” and its grammatical variants are intended to be non-limiting, such that recitation of items in a list is not to the exclusion of other like items that can be substituted or added to the listed items.

[0042] As used herein “protein”, “peptide”, and “polypeptide” are used interchangeably throughout to mean a chain of amino acids wherein each amino acid is connected to the next by a peptide bond. In some embodiments, when a chain of amino acids consists of about two to forty amino acids, the term “peptide” is used. However, the term “peptide” should not be considered limiting unless expressly indicated.

[0043] The term “antibody” is used in the broadest sense and encompasses various antibody structures, including but not limited to monoclonal antibodies, polyclonal antibodies, multispecific antibodies (such as bispecific antibodies), and antibody fragments so long as they exhibit the desired immunoprecipitating activity. As such, the term antibody includes, but is not

limited to, fragments that are capable of binding to an antigen, such as Fv, single-chain Fv (scFv), Fab, Fab', di-scFv, sdAb (single domain antibody) and (Fab')₂ (including a chemically linked F(ab')₂). Papain digestion of antibodies produces two identical antigen-binding fragments, called "Fab" fragments, each with a single antigen-binding site, and a residual "Fc" fragment. Pepsin treatment yields a F(ab')₂ fragment that has two antigen-binding sites. The term antibody also includes, but is not limited to, chimeric antibodies, humanized antibodies, and antibodies of various species such as mouse, goat, horse, sheep, chicken, etc. Furthermore, for all antibody constructs provided herein, variants having the sequences from other organisms are also contemplated, such as CDR-grafted antibodies or chimeric antibodies. Antibody fragments also include either orientation of single chain scFvs, tandem di-scFv, diabodies, tandem tri-sdcFv, minibodies, etc. Antibody fragments also include nanobodies (sdAb, an antibody having a single, monomeric domain, such as a pair of variable domains of heavy chains, without a light chain). An antibody fragment can be referred to as being a specific species in some embodiments (for example, human scFv or a mouse scFv). This denotes the sequences of at least part of the non-CDR regions, rather than the source of the construct. The antibodies are referred to by reference to name and catalog reference. The skilled artisan, holding this name and catalog information, is capable of determining the sequence of the antibody, and therefore this application encompasses any antibody having at least partial sequence of a reference antibody so long as the antibody maintains its ability to immunoaffinity enrich its antigen protein.

[0044] "Immunoaffinity enrichment" refers to any antibody-driven enrichment step. It includes, but is not limited to, methods in which a precipitate is formed, such as "immunoprecipitation."

[0045] Mass spectrometry (MS) is a technique for analysis of proteins on the basis of their mass-to-charge ratio (m/z). MS techniques generally include ionization of compounds and optional fragmentation of the resulting ions, as well as detection and analysis of the m/z of the ions and/or fragment ions followed by calculation of corresponding ionic masses. A "mass spectrometer" generally includes an ionizer and an ion detector. "Mass spectrometry," "mass spec," "mass spectroscopy," and "MS" are used interchangeably throughout.

[0046] "Targeted mass spectrometry," also referred to herein as "targeted mass spec," "targeted MS," and "tMS" increases the speed, sensitivity, and quantitative precision of mass spec analysis. Non-targeted mass spectrometry, sometimes referred to as "data-dependent

scanning,” “discovery MS,” and “dMS” and targeted mass spec are alike in that in each, analytes (proteins, small molecules, or peptides) are infused or eluted from a reversed phase column attached to a liquid chromatography instrument and converted to gas phase ions by electrospray ionization. Analytes are fragmented in the mass spec (a process known as tandem MS or MS/MS), and fragment and parent masses are used to establish the identity of the analyte. Discovery MS analyzes the entire content of the MS/MS fragmentation spectrum. In contrast, in targeted mass spectrometry, a reference spectrum is used to guide analysis to only a few selected fragment ions rather than the entire content.

[0047] “Multiple reaction monitoring,” “MRM,” “selected reaction monitoring,” and “SRM” are used interchangeably throughout to refer to a type of targeted mass spectrometry that relies on a unique scanning mode accessible on triple-quadrupole (QQQ) instruments. See, e.g., Chambers et al., *Expert Rev. Proteomics*, 1-12 (2014).

[0048] “Parallel Reaction Monitoring,” and “PRM” are used interchangeably herein to describe another type of targeted mass spec wherein the second mass analyzer used in SRM (quadrupole) is substituted by a high resolution orbitrap mass analyzer in PRM. Unlike SRM, which allows the measuring of one single transition at a given point in time, PRM allows parallel monitoring in one MS/MS spectrum. PRM also allows for the separation of ions with close m/z values (i.e., within a 10 ppm range), and may therefore allow for lower limits of detection and quantification (LOD or LLOD and LOQ or LLOQ).

[0049] To assess completion of digestion of the target protein, the number of “missed cleavages” is calculated. For example, the enzyme trypsin cuts the protein at the C-terminal side of lysine (K) or arginine (R) residues. If a peptide has a single internal K or R as well as the C-terminal K or R, that peptide has one missed cleavage. If a peptide only has the C-terminal K or R, that peptide has zero missed cleavage. If a peptide has a total of two internal K or R residues as well as the C-terminal K or R, that peptide has two missed cleaves. The same holds true for other enzymes and the residues they cleave at.

[0050] In some embodiments, a complete digestion can comprise 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% zero missed cleavages. In some embodiments, a complete digestion may comprise 0%, 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, or 10% two missed cleavages. In some embodiments, a complete digestion may comprise 0%, 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, or 10% one missed cleavages.

II. Improved Methods of Sample Preparation for Immunoaffinity Enrichment/Mass Spectrometry Methods

[0051] Improved methods for sample preparation for immunoaffinity enrichment/mass spectrometry methods include those that have the benefit of a shorter digestion time in a second enzymatic digestion, allowing for an improved user workflow and less time from initial immunoaffinity enrichment through mass spectrometry. Thus, a method for detecting one or more target protein(s) in a biological sample, comprises:

- a. enriching the target protein(s) from a biological sample by binding the target protein(s) to a solid support;
- b. fragmenting the enriched target protein(s) by:
 - i. while bound to the solid support, treating the enriched target protein(s) with a first enzymatic digestion,
 - ii. reducing and alkylating the digested target protein(s) in a single reaction vessel, and
 - iii. digesting the reduced, alkylated, and digested target protein(s) in a second enzymatic digestion, wherein optionally the second enzymatic digestion is allowed to proceed for up to 18 hours (for example up to 4 hours);
- c. detecting one or more target protein(s) in the sample.

[0052] In some embodiments, the second enzymatic digestion is allowed to proceed for up to 18 hours. In some embodiments, the second enzymatic digestion is allowed to proceed for up to 4 hours. In some embodiments, target proteins are bound to a solid support comprising a bead or a resin. In some embodiments, target proteins are bound to a solid support comprising a magnetic bead. In some embodiments, target proteins are bound to a solid support comprising an immunoaffinity bead.

[0053] In embodiments, a sample that has been enriched (including immunoaffinity enrichment) for one or more target proteins may be subjected to an elution step to separate the antibody-protein complex from a solid support. In embodiments, the elution may be an

enzymatic elution. In embodiments, an elution may be performed with a low pH/organic reagent. In embodiments, an enriched sample (including an immunoaffinity-enriched sample) may first be subjected to an enzymatic elution, and remaining antibody-protein complex bound to a substrate may subsequently be subjected to a low pH/organic elution.

[0054] In embodiments, the present disclosure provides methods of processing enriched biological samples (including immunoaffinity-enriched samples) for MS analysis. In embodiments, the samples are low amount samples (<10 microgram). In embodiments, methods described herein may be used to determine antibody epitope, specificity, and/or antigen in protein complexes.

A. Immunoaffinity Enrichment

[0055] In some embodiments, methods of immunoaffinity enriching a target protein are provided, comprising contacting a biological sample with at least one antibody. The immunoaffinity enriching method may be single-plex or multi-plex. A “single-plex” method utilizes one antibody per assay, whereas a “multi-plex” method utilizes more than one antibody per assay. Immunoaffinity enrichment may or may not comprise immunoprecipitation.

B. Reduction and Alkylation

[0056] In embodiments, the enriched protein(s) (including immunoaffinity-enriched samples) are subjected to reduction and alkylation. The enriched target proteins may be reduced and alkylated prior to fragmentation (e.g., digestion). Samples that have been reduced and alkylated may comprise modifications, such as cysteine residues. In embodiments, reducing and alkylating may take place sequentially. In embodiments, reducing and alkylating may take place in a single reaction vessel.

C. Digestion

[0057] The present method comprises two digestion steps used to fragment the enriched target protein(s) (including immunoaffinity-enriched samples): a first after enriching the target protein(s) and a second after reducing and alkylating the target proteins(s). In some embodiments, protein samples are denatured or solubilized before fragmentation.

[0058] In embodiments, the digestion is enzymatic. In embodiments, enzymatic digestion includes, but is not limited to, digestion with a protease such as, for example, trypsin,

chymotrypsin, AspN, GluC, LysC, LysN, ArgC, GluC, proteinase K, pepsin, Clostripain, Elastase, LysC/P, LysN Promisc, Protein Endopeptidase, Staph Protease or thermolysin. In some embodiments, the fragmentation protocol uses MS-grade commercially available proteases. In some embodiments, a mixture of different proteases is used (for example, trypsin and LysC). In some embodiments, the digestion is incomplete in order to see larger, overlapping peptides. In some embodiments, the antibody digestion is performed with IdeS, IdeZ, pepsin, or papain to generate large antibody domains for “middle-down” protein characterization. In some embodiments, the fragmentation protocol uses trypsin that is modified.

[0059] In some embodiments, the first digestion step is for about 5 minutes to about 4 hours, from about 10 minutes to about 1.5 hours, from about 15 minutes to about 1 hour. In some embodiments, the first digestion step is about 15 minutes, about 30 minutes, or about 1 hour, or up to about 15 minutes, up to about 30 minutes, or up to about 1 hour.

[0060] In some embodiments, the second digestion (i.e., of the reduced and alkylated target protein(s)) may proceed for about up to 4 hours, up to 3 hours, up to 2 hours, or up to 1 hour. In some embodiments, the second digestion step may proceed for about 4 hours, about 3 hours, about 2 hours, or about 1 hour. In some embodiments, the second digestion step may proceed from about 1 hour to about 4 hours.

[0061] In some embodiments, a step is included to end the digestion step. The step to end the digestion protocol may be addition of a stop solution or a step of spinning or pelleting of a sample. In some embodiments, the digestion is followed by guanidination.

[0062] In some embodiments, the fragmentation protocol is carried out in solution. An exemplary commercially available kit for performing in-solution digestion is the In-Solution Tryptic Digestion and Guanidination Kit (Thermo Fisher Cat#89895).

[0063] In some embodiments, the fragmentation protocol uses beads. In some embodiments, the fragmentation protocol comprises on-bead digestion. In some embodiments, agarose beads or Protein G beads are used. In some embodiments, magnetic beads are used.

[0064] In some embodiments, the completion of digestion is assessed by calculating the number of zero missed cleavage peptides after MS analysis or the number of zero, one, and/or two missed cleavage peptides.

D. Mass Spectrometry

[0065] The methods disclosed herein may be applied to any type of MS analysis. The disclosure is not limited by the specific equipment or analysis used. The use of any equipment with the intent of analyzing the m/z of a sample would be included in the definition of mass spectrometry. Non-limiting examples of MS analysis and/or equipment that may be used include electrospray ionization, ion mobility, time-of-flight, tandem, ion trap, MRM, SRM, MRM/SRM, PRM, and Orbitrap. The disclosure is neither limited by the type of ionizer or detector used in the MS analysis nor by the specific configuration of the MS. The disclosure is not limited to use with specific equipment or software. The disclosure is not limited to the equipment and software described in the Examples.

[0066] In embodiments, the samples may optionally be desalted prior to analysis by mass spectrometry.

[0067] In some embodiments, after fragmentation (e.g., digestion), peptide samples are analyzed by mass spectrometry (MS), and the resulting spectra are compared with theoretical spectra from known proteins to determine the peptides and proteins in a sample.

[0068] Typically, targeted MS is performed by quantifying specific unique peptides of the protein. In some embodiments, known amounts of isotope-labeled (e.g., heavy isotope-labeled) versions of these targeted peptides can be used as internal standards for absolute quantitation. In some instances, proteins of interest are not detectable even after identifying unique peptide standards. The combination of specific antibodies with specific target peptides has allowed the inventors to improve the sensitivity of detection target proteins by MS and has allowed for lower levels of detection and lower levels of quantification than previously seen.

[0069] In some embodiments, protein samples are separated using liquid chromatography before MS analysis. In some embodiments, fragmented samples are separated using liquid chromatography before MS analysis.

[0070] In some embodiments, peptides used in the MS methods described herein have limits of detection considered to be useful in clinical and research methods. In some embodiments, the peptides are detectably labelled.

[0071] In embodiments, kits are provided comprising reagents for performing methods described herein.

EXAMPLES

[0072] The following examples are provided to illustrate certain disclosed embodiments and are not to be construed as limiting the scope of this disclosure in any way.

Example 1: Low pH/organic IP elutions compared with urea-based method.

[0073] Experiments were performed to evaluate different MS sample prep methods for low pH/organic IP elutions compared with a urea-based (control) method. Various sample preparation methods were evaluated to decrease time/hands on/speed vac time by performing IP-MS using multiple antibodies mixture. The following two parameters were tested: 1) IP elution with MS compatible buffer and sequential reduction/alkylation including, as described in more details below, a) Control: Urea method; b) Adjust IP with 1M TEAB (No Urea); c) 50 mM TEAB; d) 50 mM TEAB/ 30% Acetonitrile and 2) IP elution with MS compatible buffer and one pot reduction/alkylation including a) Spin-column device with SDS; b) Spin-column device without SDS; c) PreOmics in-solution digestion kit. The following materials were used for this experiment as described below in Table 1.

[0074] **Table 1**

MATERIAL:

Material	Thermo Fisher Scientific Catalog/Product No.
Streptavidin Magnetic Beads	88817
Pierce IP Lysis Buffer	87788
Wash buffer A, Wash buffer B and Elution buffer	90408
1M triethylammonium bicarbonate (TEAB)	90114
Urea	29700
0.5M Tris[2-Carboxyethyl]phosphine (TCEP)	77720
Recombinant GFP	88899
20µg Trypsin	1862748
Iodoacetamide (IAA)	90034

[0075] **Table 2**

Target	Antibody	Amount per IP (µg)
mTOR	PA1-188	3
STAT3	13-7000	1
PAK1	71-9300	1
Ras	MA1-012	2
IQGAP1	33-8900	1
CTNNB1	71-2700	1

Target	Antibody	Amount per IP (μg)
RPTOR	42-4000	1

[0076] Experimental Protocol:

[0077] Step 1. Prepared biotinylated antibody mixture as shown in Table 2 above and mixed with 0.5mg each of HCT116 (IGF Stim:Unstim)/HEK293 lysates. Performed total of 25 IP reactions by adding appropriate amount of antibody mix (97.9μL/5mL Lysate) in 5mL low protein binding tube. Parafilmed each tube and incubated overnight at 4°C mixing on a rotator. After incubation added streptavidin magnetic beads using ratio of 1:5 (Antibody:Beads, because 10μg Antibody was used 50μL beads were added per 1mL). Washed beads with 2X volume cold IP Lysis Buffer twice. Removed wash buffer then added back original volume of IP Lysis Buffer to each tube. Pooled all IPs together in a 50mL conical tube and aliquoted into 25 1mL tubes and then added washed beads to each IP. Mixed antibody/antigen samples and rotated for 1 hour at room temperature. Washed 3 times with 500μL Wash Buffer A. Washed 2 times with 500μL Wash Buffer B.

[0078] Step 2. The following MS sample prep solutions were prepared: 50mM TEAB – Diluted 1M Trimethylammonium Bicarbonate (TEAB) (PN#90114) to 50mM by adding 0.5mL TEAB in 9.5mL MS grade water, pH 8.5; Denaturation Solution (6M Urea, recombinant GFP) – Added 360mgs Urea to 675μL 50mM TEAB and vortex (exothermic reaction). Added 400μL of this solution to the GFP standard tube 50ng; 50mM TEAB in 30% ACN – Added 500μL 100mM TEAB to 300μL ACN and 200μL MS grade water; 10mM TCEP – Diluted 10μL 0.5M TCEP (PN#77720) with 490μL 50mM TEAB, pH8.5; 5mM TCEP – Diluted 5μL 0.5M TCEP with 495μL 50mM TEAB, pH8.5; 0.5mM Iodoacetamide (IAA) – Dissolved 9.3mgs No-weigh IAA (PN#90034) in 100μL MS-Grade water (Protect from light); 0.1μg/μL Trypsin Stock – Dissolved 20μg Trypsin protease (PN#90057) in 200μL 0.1% Acetic acid, stored as 30μL aliquots at -80°C.

[0079] Step 3. Mass Spec Sample Prep Methods tested included. Samples 1-3 = 6M Urea; samples 4-6 = No Urea – 50mM TEAB + 30% ACN; samples 7-9 = Preomics Kit; samples 10 – 12 = LysC Elution from Bead; samples 13 – 15 = Trypsin Elution from Bead; samples 16 – 18 = LysC + Trypsin Elution from Bead; samples 19 – 21 = On bead Digestion – Remove from bead then Trypsin; samples 22 – 25 = On bead Digestion overnight – Kept on bead then Trypsin. The following elution methods were tested. Samples 1-9 were eluted with 220μL IP-MS Elution

buffer (0.5% formic acid; 30% acetonitrile solution) Vortexed well, quick spin and let sit for 10mins at room temperature, vortexed and quick spin and put on magnet then removed as much as possible into 2, 2 mL tubes to pool the elutes together. Spun pooled lysates at 15000 x g for 2 mins and put on magnet then remove eluted and divided into 205µL per tube into 10 different tubes labeled 1-9. Dried the samples in speed vac > 1hr (35°C) – checking every 30 mins vortexing and drying until done; did not see any pellet. Samples 10-12 were eluted by adding 1µg LysC (PN#90051) in 100µL 50mM TEAB to each sample. This solution was made by adding 5µL of 0.2µg/µl solution + 95µl 50mM TEAB per sample. Samples 13-15 were eluted by adding 1µg Trypsin (PN#1862748) in 100µL 50mM TEAB. This solution was made by adding 5µL of 0.2µg/µl solution + 95µl 50mM TEAB per sample. Samples 16-18 were eluted by adding 1µg LysC/Trypsin in 100µL 50mM TEAB. This solution was made by adding 5µL of 0.2µg/µl LysC/Trypsin solution + 95µl 50mM TEAB per sample. All samples 10-18 were followed by incubation at 37°C 800rpm for 1.5 hour. Then beads were collected on magnet and 90µl of supernatant was removed to a clean tube. This was followed by addition of 2µl 25ng/µl recombinant GFP and 0.92µl 0.5M TCEP and then incubated at 60°C for 30mins. For Samples 19-24, prepared 60µl of Trypsin plus 540µl 50mM TEAB and added 100µl of this solution to each and incubated at 37°C at 800 rpm for 1hour. After 1 hour of incubation 1µl 0.5M TCEP and 2µl 25ng/µl GFP were added and incubated for 30 mins at 37°C at 800 rpm.

[0080] Step 4. The sample prep continued for trypsin digestion as described below: For Samples 1-3 - Suspended the dried sample in 10µL 6M Urea/TEAB/GFP solution and vortexed for 30 seconds followed by addition of 10µL 10mM TCEP mixed and incubated at 35°C mixing at 1000rpm for 30mins; For Samples 4-6 - Resuspended in 20µL of solution containing 50mM TEAB/30%ACN, 50ng of rGFP. Incubated at 60°C for 30mins at 500rpm. For Samples 7-9 - Kept dried down pellet at -20°C for Preomics kit the next day.

[0081] Step 5. Performed alkylation of peptides using IAA as described below: For Samples 1-6 - Added 1µL IAA solution mix; For Samples 10-18 - Added 4.6µL IAA; For Samples 19-24: Added 5.15 µL IAA. Incubate all 30mins RT protected from light.

[0082] Step 6. Performed trypsin digestion as described below: For Samples 1-6 - Immediately added 45µL 50mM TEAB, pH 8.5. Prepared 20ng/µL trypsin solution by adding 120µL 50mM TEAB solution to 30µL aliquots of 0.1µg/µL Trypsin Stock and then added 10µL of this to samples 1-18. Volume at this point was 76µL (Samples 1-6), 107.676µL (Samples 10-

18). For samples 19-21, removed 92μL from beads then added the 10μL of trypsin. For samples 22-24, kept on bead for trypsin digestion. Incubated all samples at 37°C for 18.5hrs at 500rpm.

[0083] Step 7. Prepared a mixture of 0.2% Formic Acid (FA), 4% Acetonitrile (ACN) to resuspend the dried samples by adding 940μL MS grade water, 20μL 10%FA and 40μL of 100% ACN. Removed 92μL of samples 22-24 from the beads. Acidified the samples by adding 3.5μL 10%TFA (pH< 3). Added 4.5μL of 10% TFA to acidify samples 10-24. Centrifuged all samples at 15,000 x g for 2mins. Removed 65μL of samples 1-6, 90μL of samples 10-18 and 82μL of Samples 19-24 to a new low protein binding tube. Dried down the samples for about 1hr. Added 0.2% FA and 4% ACN to each tube as described below: 13μL for samples 1-3 and 17μL for all remaining samples and vortexed to mix. Stored all samples at -20°C before nanoLC-MS/MS analysis.

[0084] As shown in Figure 3, the urea method worked best compared to no urea and device-assisted methods.

Example 2: Enzyme elutions compared with low pH/organic IP elution method.

[0085] Another experiment was performed to evaluate various sample prep methods by performing IP-MS using QC Mix Antibodies. Sequential reduction and alkylation (with/without beads) was followed by a second trypsin digestion overnight.

[0086] Step 1. Prepared QC Mix for 15 IPs as shown in Table 2.

[0087] Step 2. Used 0.5mg each of HCT116 (IGF Stim:Unstim)/HEK293 lysates per IP. Set up 15 IP reactions by adding appropriate amount of antibody mix to 1mg of lysate per IP. Used parafilm to cover the tube caps and rotated overnight on 4°C.

[0088] Step 3. Used 50μL beads for each IP using ratio of 1:5 for antibody amount to bead volume. Washed beads with 2X volume cold IP Lysis Buffer twice. Added 1mL of antigen-antibody mixture to 50μL beads and rotated for 1 hr at room temperature. Washed 3 times with 500μL of Wash Buffer A followed by 2 times with 500μL of Wash Buffer B. Evaluated the following methods for Mass Spec Sample Prep: samples 1-3 = elution buffer followed by Urea in-solution digestion; samples 4-6 = Trypsin Elution from Bead; samples 7-9 = LysC + Trypsin Elution from Bead; samples 10 – 12 = On bead Digestion overnight – Keep on bead then Trypsin; samples 13 – 15 = On bead Digestion – removed from bead then Trypsin.

[0089] Step 4. Eluted Samples 1-3 with 220 μ L IP-MS Elution buffer for 10mins at room temperature. Placed on magnet and removed 220 μ L to a new 1.5 mL low-protein binding tubes to pool the elutes together. Centrifuged the pooled samples at 15000 x g for 2 mins and put on magnet then pull elute divide into 205 μ L per tube into 3 different tubes labeled 1-3. Dried the samples in speed vac > 1hr (35°C). Eluted samples 4-6 by adding 1 μ g Trypsin in 100 μ L 50mM TEAB and incubated at 37°C 800rpm for 1.5 hours. After incubation, put on magnet to remove beads and took 90 μ L supernatant followed by addition of 2 μ L 25ng/ μ L GFP and 0.92 μ L 0.5M TCEP and incubate 60°C for 30 minutes. Eluted samples 7-9 by adding 1 μ g LysC/Trypsin in 100 μ L 50mM TEAB, pH 8.5 and incubated at 37°C 800rpm for 1.5 hours. Eluted samples 10-15 by adding 1 μ g of Trypsin in 50mM TEAB buffer and incubated at 37°C at 800 rpm for 1 hour. After 1 hr of incubation, added 1 μ L 0.5M TCEP and 2 μ L 25ng/ μ L rGFP and incubated for 30 mins at 37°C at 800 rpm.

[0090] Step 5. Prepared the following solution for the next step of sample prep:
 Denaturation Solution – 6M Urea + GFP - Used 360mgs aliquots of Urea by adding 675 μ L 50mM TEAB and vortex (exothermic reaction). Added 400 μ L of this solution to the GFP standard tubes 50ng; 10mM TCEP – Diluted 10 μ L 0.5M TCEP with 490 μ L 50mM TEAB, pH8.5; 5mM TCEP – Diluted 5 μ L 0.5M TCEP with 495 μ L 50mM TEAB, pH8.5; 0.5mM IAA – Dissolved No-weigh IAA 9.3mgs in 100 μ L MS-Grade water (Protect from light); 0.1 μ g/ μ L Trypsin Stock – Dissolved 20 μ g Trypsin protease (P#90057) in 200 μ L 0.1% Acetic acid, stored as 30 μ L aliquots at -80°C. 20ng/ μ L trypsin working solution (prepared just before use) – Added 120 μ L 50mM TEAB solution to 30 μ L aliquots of 0.1 μ g/ μ L trypsin stock; 0.2% Formic Acid (FA), 4% Acetonitrile (ACN) – Added 940 μ L MS grade water to 20 μ L 10% FA and 40 μ L of 100% ACN.

[0091] Step 6. For samples 1-3, added 10 μ L 6M Urea/50mM TEAB /rGFP solution to dried samples and vortexed for 30 seconds. Added 10 μ L 10mM TCEP mix and incubate at 35°C mixing at 1000rpm for 30mins. Added 1 μ L IAA for samples 1-3, 4.6 μ L IAA for samples 4-9, 5.15 μ L IAA for samples 10-15. Incubated all samples for 30 minutes at room temperature protected from light. Added 45 μ L 50mM TEAB, pH 8.5 to samples 1-3. For samples 13-15, removed 92 μ L supernatant from beads on magnet. Added 10 μ L of 20ng/ μ L trypsin working solution to each of samples 1-15 and incubated at 37°C for 18.5 hours at 500rpm. Removed 92 μ L of each sample 10-12 from the beads. Acidified all the samples by adding 5 μ L 10%TFA,

checked pH (pH<3). Centrifuged at 15,000 x g for 2mins. Removed the following volumes for each sample groups: 65µL for samples 1-3;

[0092] 90µL for samples 4-9; 82µL for samples 10-15. Speed-vac dried the samples for about 1 hour. Added 0.2% FA and 4% ACN solution to each tube: 13µL for samples 1-3 and 17µL for all remaining samples. Stored all samples at -20°C before nanoLC-MS/MS analysis.

[0093] Figure 4 shows the results of IP elution with enzyme and sequential reduction/alkylation. The results shown included the following: a) LysC elution b) Trypsin elution c) LysC/Trypsin elution d) Trypsin elution with On-bead reduction/alkylation (No Bead) e) Trypsin elution with On-bead reduction/alkylation/digestion.

[0094] As shown in Figure 4, the enzyme elution method showed better recovery of most of the targets compared to IP-MS elution buffer-based methods.

[0095] Significant reduction in antibody leaching was observed with enzyme elution method. As shown in Figure 5, enzyme elution showed 1 to 2 orders of magnitude lower levels of IgGs compared to IP-MS elution buffer methods. Lower leaching of antibodies was found with the removal of bead after trypsin elution.

Example 3: Single pot reduction/alkylation using trypsin and/or LysC enzyme elution method.

[0096] An additional experiment was performed to evaluate various sample prep methods to decrease time/hands on/speed vac time by performing IP-MS using QC Mix Antibodies. The following were evaluated for IP elution with enzyme and one pot reduction/alkylation: a) LysC or Trypsin or LysC/Trypsin elution with one pot reduction/alkylation; b) LysC or Trypsin or LysC/Trypsin elution with one pot reduction/alkylation with beads; c) LysC or Trypsin or LysC/Trypsin elution with one pot reduction/alkylation and digestion with beads.

[0097] Step 1. Prepared QC Mix for 15 IPs as shown in table 2 except replacement of Ras Antibody (PN#33-7200). Used 0.5mg each of HCT116 (IGF Stim:Unstim)/HEK293 lysates for each IP reaction. Sealed the tubes with Parafilm and rotated overnight on 4°C.

[0098] Step 2. Used 50µL beads for each IP using ratio of 1:5 for antibody amount to bead volume. Washed beads with 2X volume cold IP Lysis Buffer twice. Added 1mL of antigen-antibody mixture to 50µL beads and rotated for 1 hr at room temperature. Washed 3 times with 500µL of Wash Buffer A followed by 2 times with 500µL of Wash Buffer B. Evaluated the following methods for Mass Spec Sample Prep: Samples 1-3 = Trypsin elution from bead;

samples 4-6 = Trypsin elution – Single Pot (SP) reduction/alkylation; samples 7-9 = On bead digestion – Removed from bead then Trypsin (No Bead); samples 10 – 12 = On bead digestion – Removed from bead then Trypsin – Single Pot reduction/alkylation (No bead-SP); samples 13 – 15 = Control (Urea).

[0099] Step 3. Eluted samples 13-15 with 220 μ L IP-MS Elution buffer, Vortexed well, quick spin and incubated for 10 minutes at room temperature, vortexed and quick spin and put on magnet then removed as much as possible into 2 mL tubes to pool the elutes together.

Centrifuged the pooled samples at 15000 x g for 2 minutes and put on magnet then pull elute divided into 205 μ L per tube into different tubes labeled 13-15. Dried the samples in speed vac for about 1hr (35°C). Eluted samples 1-12 by adding 1 μ g Trypsin in 100 μ L 50mM TEAB and incubated at 37°C 800rpm for 1 hour. After incubation, put samples 1-6 on magnet to remove beads and took 90 μ L supernatant followed by addition of 2 μ L 25ng/ μ L GFP and 0.92 μ L 0.5M TCEP. Incubated samples 1-3 at 60°C for 30 minutes and samples 4-6 at 95°C for 5 minutes. For samples 7-9, added 1 μ L 0.5M TCEP and 2 μ L 25ng/ μ L GFP and incubated for 30 mins at 37°C at 800 rpm. For samples 10-12, added 2 μ L 25ng/ μ L GFP and 25 μ L one pot reduction/alkylation solution (Final 50mM TEAB, pH 8.5; 10mM TCEP; 20mM chloroacetylamide (CLAA) (Thermo Fisher Scientific; PN# A39270)) and incubated at 95°C 5 mins.

[00100] Step 4. Prepared the following solution for the next step of sample prep:
Denaturation Solution – 6M Urea + GFP - Used 360mgs aliquots of Urea by adding 675 μ L 50mM TEAB and vortex (exothermic reaction). Added 400 μ L of this solution to the GFP standard tubes 50ng; 10mM TCEP – Diluted 10 μ L 0.5M TCEP with 490 μ L 50mM TEAB, pH8.5; 5mM TCEP – Diluted 5 μ L 0.5M TCEP with 495 μ L 50mM TEAB, pH8.5; 0.5mM IAA – Dissolved No-weigh IAA 9.3mgs in 100 μ L MS-Grade water (Protect from light); 0.1 μ g/ μ L Trypsin Stock – Dissolved 20 μ g Trypsin protease (P#90057) in 200 μ L 0.1% Acetic acid, stored as 30 μ L aliquots at -80°C. 20ng/ μ L trypsin working solution (prepared just before use) – Added 120 μ L 50mM TEAB solution to 30 μ L aliquots of 0.1 μ g/ μ L trypsin stock; 0.2% Formic Acid (FA), 4% Acetonitrile (ACN) – Added 940 μ L MS grade water to 20 μ L 10% FA and 40 μ L of 100% ACN.

[00101] Step 5. For samples 13-15, added 10 μ L 6M Urea/50mM TEAB /rGFP solution to dried samples and vortexed for 30 seconds. Added 10 μ L 10mM TCEP mix and incubate at 35°C mixing at 1000rpm for 30mins. Added 1 μ L IAA for samples 13-15, 4.6 μ L IAA for samples 1-3,

4.9 μL IAA for samples 7-9. Incubated all samples for 30 minutes at room temperature protected from light. Added 45 μL 50mM TEAB, pH 8.5 to samples 13-15. For samples 7-12, removed supernatant from beads on magnet. Added 10 μL of 20ng/ μL trypsin working solution to each of samples 1-15 and incubated at 37°C for 18.5 hours at 500rpm. Acidified all the samples by adding 5 μL 10%TFA, checked pH (pH<3). Centrifuged at 15,000 x g for 2mins. Removed the following volumes for each sample groups: 96 μL for samples 1-3 and samples 7-9; 112 μL for samples 4-6 and samples 10-12. Speed-vac dried the samples for about 1 hour. Added 0.2% FA and 4% ACN solution to each tube: 17 μL for samples 1-12 and 13 μL for samples 13-15. Stored all samples at -20°C before nanoLC-MS/MS analysis.

[00102] As shown in Figure 6, enzyme elution with the single pot reduction/alkylation showed better recovery of targets from QC mixture.

Example 4: IP-MS using QC Mix Antibodies

[00103] An additional experiment was conducted to evaluate various sample prep methods to further validate enzyme elutions by performing IP-MS using QC Mix Antibodies.

[00104] Step 1. Prepared QC Mix for 12 IPs as shown in table 2 except replacement of Ras Antibody (PN#33-7200). Used 0.5mg each of HCT116 (IGF Stim:Unstim)/HEK293 lysates for each IP reaction. Sealed the tubes with Parafilm and rotated overnight on 4°C.

[00105] Step 2. Used 50 μL beads for each IP using ratio of 1:5 for antibody amount to bead volume. Washed beads with 2X volume cold IP Lysis Buffer twice. Added 1mL of antigen-antibody mixture to 50 μL beads and rotated for 1 hr at room temperature. Washed 3 times with 500 μL of Wash Buffer A followed by 2 times with 500 μL of Wash Buffer B. Evaluated the following methods for Mass Spec Sample Prep: Samples 1-3 = Control (Urea); Samples 4-6 = Trypsin elution from bead; samples 7-9 = Trypsin elution from beads– Single Pot (SP) reduction/alkylation; samples 10-12 = On bead digestion – Removed from bead then Trypsin.

[00106] Step 3. Eluted samples 1-3 with 220 μL IP-MS Elution buffer, Vortexed well, quick spin and incubated for 10 minutes at room temperature, vortexed and quick spin and put on magnet then removed as much as possible into 1.5 mL tubes to pool the elutes together. Centrifuged the pooled samples at 15000 x g for 2 minutes and put on magnet then aliquoted into 205 μL per tube into different tubes labeled 1-3. Dried the samples in speed vac for about 1hr (35°C). Eluted samples 4-12 by adding 1 μg Trypsin in 100 μL 50mM TEAB and incubated at

37°C 800rpm for 1 hour. After incubation, put samples 4-6 on magnet to remove beads and took 90µl supernatant followed by addition of 2µl 25ng/µl GFP and 0.92µl 0.5M TCEP. Incubated samples 4-6 at 60°C for 30 minutes. After incubation, put samples 7-9 on magnet to remove beads and took 90µl supernatant followed by addition of 2µl 25ng/µl GFP and 25µL one pot reduction/alkylation solution (25µL one pot reduction/alkylation solution (Final 50mM TEAB, pH 8.5; 10mM TCEP; 20mM chloroacetylamine (CLAA) (Thermo Fisher Scientific; PN# A39270)) and incubated at 95°C for 5 minutes. For samples 10-12, added 1µl 0.5M TCEP and 2µl 25ng/µl GFP and incubated for 30 mins at 37°C at 800 rpm.

[00107] Step 4. Prepared the following solution for the next step of sample prep:
 Denaturation Solution – 6M Urea + GFP - Used 360mgs aliquots of Urea by adding 675µL 50mM TEAB and vortex (exothermic reaction). Added 400µL of this solution to the GFP standard tubes 50ng; 10mM TCEP – Diluted 10µL 0.5M TCEP with 490µL 50mM TEAB, pH8.5; 5mM TCEP – Diluted 5µL 0.5M TCEP with 495µL 50mM TEAB, pH8.5; 0.5mM IAA – Dissolved No-weigh IAA 9.3mgs in 100µL MS-Grade water (Protect from light); 0.1µg/µL Trypsin Stock – Dissolved 20µg Trypsin protease (P#90057) in 200µL 0.1% Acetic acid, stored as 30µL aliquots at -80°C. 20ng/µL trypsin working solution (prepared just before use) – Added 120µL 50mM TEAB solution to 30µL aliquots of 0.1µg/µL trypsin stock; 0.2% Formic Acid (FA), 4% Acetonitrile (ACN) – Added 940µL MS grade water to 20µL 10% FA and 40µL of 100% ACN.

[00108] Step 5. For samples 1-3, added 10µL 6M Urea/50mM TEAB /rGFP solution to dried samples and vortexed for 30 seconds. Added 10µL 10mM TCEP mix and incubate at 35°C mixing at 1000rpm for 30mins. Added 1µL IAA for samples 1-3, 4.6µL IAA for samples 4-6, 4.9 µL IAA for samples 10-12. Incubated all samples for 30 minutes at room temperature protected from light. Added 45µL 50mM TEAB, pH 8.5 to samples 13-15. For samples 7-12, removed supernatant from beads on magnet. Added 10µL of 20ng/µL trypsin working solution to each of samples 1-12 and incubated at 37°C for 18.5 hours at 500rpm. Acidified all the samples by adding 5µL 10%TFA, checked pH (pH<3). Centrifuged at 15,000 x g for 2mins. Removed the following volumes for each sample groups: 68µL for samples 1-3; 96µL for samples 4-6 and samples 10-12; 112µL for samples 7-9. Speed-vac dried the samples for about 1 hour. Added 0.2% FA and 4% ACN solution to each tube: 13µL for samples 1-3 and 17µL for samples 4-12. Stored all samples at -20°C before nanoLC-MS/MS analysis.

[00109] The results are shown in Figure 7. Trypsin elution with single pot (SP) reduction/alkylation showed equal or better recovery of 9 target proteins compared to Urea, Trypsin elution with sequential reduction/alkylation and on bead trypsin digestion.

Example 5: IP-MS using Akt Phospho Mix Antibodies

[00110] An experiment was performed to evaluate sample prep methods by performing IP-MS using Akt Phospho Mix Antibodies (Thermo Fisher Scientific, PN# A40086).

[00111] Step 1. Prepared AKT Phospho Antibody mixture for 9 IPs. Used 1mg each of MCF7 Stim (+hIGF) lysate for each IP reaction. Sealed the tubes with Parafilm and rotated overnight on 4°C.

[00112] Step 2. Used 55µL beads for each IP using ratio of 1:5 for antibody amount to bead volume. Washed beads with 2X volume cold IP Lysis Buffer twice. Added 1mL of antigen-antibody mixture to 55µL beads and rotated for 1 hr at room temperature. Washed 3 times with 500µL of Wash Buffer A followed by 2 times with 500µL of Wash Buffer B. Evaluated the following methods for Mass Spec Sample Prep: Samples 1-3 = Control (Urea); Samples 4-6 = Trypsin elution from bead; samples 7-9 = Trypsin elution from beads– Single Pot (SP) reduction/alkylation.

[00113] Step 3. Eluted samples 1-3 with 220µL IP-MS Elution buffer, Vortexed well, quick spin and incubated for 10 minutes at room temperature, vortexed and quick spin and put on magnet then removed as much as possible into 1.5 mL tubes to pool the elutes together. Centrifuged the pooled samples at 15000 x g for 2 minutes and put on magnet then aliquoted into 205µL per tube into different tubes labeled 1-3. Dried the samples in speed vac for about 1hr (35°C). Eluted samples 4-9 by adding 1µg Trypsin in 100µL 50mM TEAB and incubated at 37°C 800rpm for 1 hour. After incubation, put samples 4-6 on magnet to remove beads and took 90µl supernatant followed by addition of 2µl 25ng/µl GFP and 0.92µl 0.5M TCEP. Incubated samples 4-6 at 60°C for 30 minutes. After incubation, put samples 7-9 on magnet to remove beads and took 90µl supernatant followed by addition of 2µl 25ng/µl GFP and 25µL one pot reduction/alkylation solution (25µL one pot reduction/alkylation solution (Final 50mM TEAB, pH 8.5; 10mM TCEP; 20mM chloroacetyamide (CLAA) (Thermo Fisher Scientific; PN# A39270)) and incubated at 95°C for 5 minutes.

[00114] Step 4. Prepared the following solution for the next step of sample prep:

Denaturation Solution – 6M Urea + GFP - Used 360mgs aliquots of Urea by adding 675µL 50mM TEAB and vortex (exothermic reaction). Added 400µL of this solution to the GFP standard tubes 50ng; 10mM TCEP – Diluted 10µL 0.5M TCEP with 490µL 50mM TEAB, pH8.5; 5mM TCEP – Diluted 5µL 0.5M TCEP with 495µL 50mM TEAB, pH8.5; 0.5mM IAA – Dissolved No-weigh IAA 9.3mgs in 100µL MS-Grade water (Protect from light); 0.2µg/µL Trypsin Stock – Dissolved 20µg Trypsin protease (P#90057) in 100µL 0.1% Acetic acid, stored as 30µL aliquots at -80°C. 40ng/µL trypsin working solution (prepared just before use) – Added 120µL 50mM TEAB solution to 30µL aliquots of 0.2µg/µL trypsin stock; 0.2% Formic Acid (FA), 4% Acetonitrile (ACN) – Added 940µL MS grade water to 20µL 10% FA and 40µL of 100% ACN.

[00115] Step 5. For samples 1-3, added 10µL 6M Urea/50mM TEAB /rGFP solution to dried samples and vortexed for 30 seconds. Added 10µL 10mM TCEP mix and incubate at 35°C mixing at 1000rpm for 30mins. Added 1µL IAA for samples 1-3 and 4.6µL IAA for samples 4-6. Incubated all samples for 30 minutes at room temperature protected from light. Added 45µL 50mM TEAB, pH 8.5 to samples 13-15. For samples 7-9, removed supernatant from beads on magnet. Added 10µL of 20ng/µL trypsin working solution to each of samples 1-9 and incubated at 37°C for 18.5 hours at 500rpm. Acidified all the samples by adding 5µL 10%TFA, checked pH (pH<3). Centrifuged at 15,000 x g for 2mins. Removed the following volumes for each sample groups: 68µL for samples 1-3; 96µL for samples 4-6; 112µL for samples 7-9. Speed-vac dried the samples for about 1 hour. Added 0.2% FA and 4% ACN solution to each tube: 13µL for samples 1-3 and 17µL for samples 4-9. Stored all samples at -20°C before nanoLC-MS/MS analysis.

[00116] The results are shown in Figure 8. Trypsin elution with single pot (SP) reduction/alkylation showed better recovery of 9 of 11 AKT pathway phosphorylated prpteins compared to control Urea and Trypsin elution with sequential reduction/alkylation methods.

[00117] Table 3 shows day to day coefficient of variation (CV)s are better for enzyme elution (i.e., less than 25%).

[00118] **Table 3**

% CV between different experiments (days)			
Target	Urea	TrypE	TrypE_SP
pAKT2	35%	4%	13%
pAKT1	57%	22%	9%
pPRAS40	15%	6%	8%
pmTOR	38%	10%	7%
pGSK3a	22%	7%	4%
pIGF1R	34%	2%	16%
pIRS1	20%	12%	40%
pp70S6K	15%	16%	16%
pGSK3b	24%	15%	5%
pTSC2	15%	20%	5%
pPTEN	25%	141%	61%

[00119] In view of the above, the optimal condition was found to be trypsin elution from the beads, combined with single pot reduction/alkylation followed by overnight second trypsin digestion.

Example 6: Optimizing Time for Second Trypsin Digestion

[00120] Experiments were set up to test different digestion times for the second trypsin digestion for MS sample prep optimization. The emphasis was to compare the overnight second trypsin digestion with shorter digestion times with purpose to produce a one-day sample prep method.

Experimental Protocol:

[00121] Step 1. Materials used are same as listed in Table 1. AKT Phospho Multiplex Antibody mix (PN#A40086) was used and mixed with 0.5mg each HCT116 (+/-) lysates. Performed total of 16 IP reactions (2 replicates for each condition) by adding appropriate amount

of antibody mix in 1.5mL low –protein binding tube. Parafilm each tube and incubated overnight at 4°C mixing on a rotator. After incubation added streptavidin magnetic beads using ratio of 1:5 (Antibody:Beads, Since 14µg Antibody was used 70µL beads were added per 1mL). Washed beads with 2X volume cold IP Lysis Buffer twice. Removed wash buffer then added back original volume of IP Lysis Buffer to each tube then added washed beads to each IP. Mixed antibody/antigen samples and rotated for 1 hour at room temperature. Washed 3 times with 500µL Wash Buffer A. Washed 2 times with 500µL Wash Buffer B.

[00122] Step 2. The following MS sample prep solutions were prepared: 50mM TEAB – Diluted 1M Trimethylammonium Bicarbonate (TEAB) (PN#90114) to 50mM by adding 0.5mL TEAB in 9.5mL MS grade water, pH 8.5; Single Pot Reduction Alkylation – Final 50 mM TEAB pH 8.5; 10 mM TCEP; 20 mM chloroacetamide (CLAA) (Thermo Fisher Scientific; PN# A39270); 0.2µg/µL Trypsin Stock – Dissolved 20µg Trypsin protease (PN#90057) in 100µL 0.1% Acetic acid; CaCl₂ solution - Add 13.78 mg CaCl₂ in 150µL Trypsin + 50mM TEAB Solution; 0.2% Formic Acid (FA), 4% Acetonitrile (ACN) - Add 940µL MS grade water, 20µL 10%FA and 40µL of 100% ACN.

[00123] Step 3. Mass Spec Sample Prep Methods tested included. Samples 1-2 = Control – Regular overnight trypsin digestion at 37°C; samples 3-4 = 1 hour trypsin digestion at 37°C; samples 5-6 = 2 hour trypsin digestion at 37°C; samples 7-8 = 3 hour trypsin digestion at 37°C; samples 9-10 = 4 hour trypsin digestion at 37°C; samples 11-12 = 1 hour trypsin digestion at 60°C; samples 13-14 = 2 hour trypsin digestion at 60°C; and samples 15-16 = 2 hour trypsin digestion at 60°C + 50mM CaCl₂. All reactions were performed in Thermomixer set at stated temperature and 800 rpm.

[00124] Step 4. The sample prep continued for trypsin elution by adding 1µg trypsin in 100µl 50 mM TEAB to each sample and incubated for 1 hour at 37°C in a thermomixer shaking at 800 rpm. After 1 HR incubation, tubes were placed onto magnet to remove beads. 90µL of supernatant was removed and 2µL 25 ng/µL GFP and 25µl single pot reduction/alkylation solution were added to each reaction and incubated at 95°C for 5 mins.

[00125] Step 5. After Reduction/Alkylation added trypsin digestion enzymes as stated in step 3 and incubated at stated times and temperatures for digestion reactions.

[00126] Step 6. Acidified the samples by adding 3.5µL 10%TFA (pH< 3) to all samples except 15-16 where 4.5µL of 10% TFA + 1µL 25% TFA was added acidify samples.

Centrifuged all samples at 15,000 x g for 2mins and then removed 112 μ L of samples to a clean 1.5 ml low protein binding tube. Dried down the samples for about 1hr in speed vac. Added 17 μ L of 0.2% FA and 4% ACN to each tube. Stored all samples at -20°C before nanoLC-MS/MS analysis.

[00127] Results of three experiments are summarized in Table 4 below.

[00128] **Table 4**

Exp 1 n=3 (Operator A)	Exp 2 n=2 (Operator A)	Exp 3 n=3 (Operator B)
1 Hour at 37°C	1 Hour at 37°C 1 Hour at 60°C	1 Hour at 37°C
2 Hours at 37°C	2 Hours at 37°C 2 Hours at 60°C 2 Hours at 60°C +50mM CaCl ₂	2 Hours at 37°C
3 Hours at 37°C	3 Hours at 37°C	3 Hours at 37°C
4 Hours at 37°C	4 Hours at 37°C	4 Hours at 37°C
Overnight (18.5Hrs) at 37°C (Control)	Overnight (18.5Hrs) at 37°C (Control)	Overnight (18.5Hrs) at 37°C (Control)

[00129] As shown in Figures 9-11, 1, 2, and 3 hours showed very comparable data compared to overnight digestion.

[00130] Figures 9A and 9B show average top peptide areas from Experiment 1 above. Samples digested for 1/2/3 hours give equivalent or better intensities for all the targets as compared to those digested overnight. Samples digested for 4 hours showed lower intensities. PD 1.4 and 2.2 results correlate.

[00131] Figure 10A shows average peptide areas for Experiment 2 above. Samples digested for 1/2/3 hours give equivalent or better intensities for all the targets as compared to those digested overnight. Samples digested for 4 hours showed comparable intensities.

[00132] Figure 10B shows average peptide areas for Experiment 3 above. Except for IQGAP1, all the targets meet the specs for all conditions.

[00133] Figure 11 shows %CV of peptide area for three experiments shown in Table 4 above. Overall <25% CV was observed with 1/2/3/4 hrs or O/N digestion time points.

[00134] Increasing the temperature to 60°C and addition of CaCl₂ did not improve results.

[00135] Targeted MS analysis was performed to evaluate recovery of multiple unique peptides for each target protein across different digestion times. The results are shown in Figures 12A-F. Low recovery of most peptides for CTNNB1 (Figure 12E) and IQGAP1 (Figure 12 F) was observed with different digestion time points.

Example 7: Effects of trypsin amount and testing with LysC in second digestion.

[00136] Experiments were performed to test different trypsin amounts with different digestion times with and without LysC for MS sample prep optimization. IP was followed by MS (Single pot reduction/alkylation) and modified trypsin digestion step. Table 5 below shows the experimental design.

[00137] **Table 5**

One Hour at 37°C	Two Hour at 37°C	Overnight at 37°C
200ng Trypsin (1:40)	200ng Trypsin (1:40)	200ng Trypsin (1:40)
600ng Trypsin (1:13)	600ng Trypsin (1:13)	----
300ng Trypsin + 300ng LysC (1:13)	300ng Trypsin + 300ng LysC (1:13)	
800ng Trypsin (1:10)	800ng Trypsin (1:10)	

[00138] Experimental Protocol:

[00139] Step 1. Materials used are same as listed in Table 1. Prepared biotinylated antibody mixture as shown in Table 2 above and mixed with 0.5mg each of HCT116 (IGF Stim:Unstim)/HEK293 lysates. Performed total of 24 IP reactions by adding appropriate amount of antibody mix in 1.5 mL low protein binding tube. Parafilm each tube and incubated overnight at 4°C mixing on a rotator. After incubation added streptavidin magnetic beads using ratio of 1:5 (Antibody:Beads, because 10µg Antibody was used 50µL beads were added per 1mL). Washed beads with 2X volume cold IP Lysis Buffer twice. Removed wash buffer then added back original volume of IP Lysis Buffer to each tube. Pooled all IPs together in a 50mL conical tube and aliquoted into 25 1mL tubes and then added washed beads to each IP. Mixed

antibody/antigen samples and rotated for 1 hour at room temperature. Washed 3 times with 500µL Wash Buffer A. Washed 2 times with 500µL Wash Buffer B.

[00140] Step 2. The following MS sample prep solutions were prepared: 50mM TEAB – Diluted 1M Trimethylammonium Bicarbonate (TEAB) (PN#90114) to 50mM by adding 0.5mL TEAB in 9.5mL MS grade water, pH 8.5; Single Pot Reduction Alkylation – Final 50 mM TEAB pH 8.5; 10 mM TCEP; 20 mM chloroacetamide (CLAA) (Thermo Fisher Scientific; PN# A39270); 0.2µg/µL Trypsin Stock – Dissolved 20µg Trypsin protease (PN#90057) in 100µL 0.1% Acetic acid; Various trypsin stocks – 20ng/µL = 30µL trypsin stock + 120µL 50 mM TEAB solution then add 10µL to sample 1, 2, 9, 10, 17, 18, 19 – 24; 60ng/µL = 90µL trypsin stock + 60µL 50mM TEAB solution then add 10µL to samples 3, 4, 11 and 12; 80ng/µL = 120µL trypsin stock + 30µL 50mM TEAB solution then add 10µL to samples 7, 8, 15 and 16; 60ng/µL Trypsin/LysC stock- Add 200µL 50mM Acetic acid to 20µg trypsin/LysC then make solution = 90µL trypsin/LysC stock + 60µL 50mM TEAB solution then add 10µL to samples 5, 6, 13 and 14; 0.2% Formic Acid (FA), 4% Acetonitrile (ACN) - Add 940µL MS grade water, 20µL 10%FA and 40µL of 100% ACN.

[00141] Step 3. Mass Spec Sample Prep Methods tested included. Samples 1-2 = 1 hr trypsin digestion at 37°C, 200ng trypsin (~1:40, assuming we have 6-8µg peptides); samples 3-4 = 1 hr trypsin digestion at 37°C, 600ng trypsin (~1:10); samples 5-6 = 1 hr trypsin digestion at 37°C, 300ng trypsin + 300ng LysC (~1:10); samples 7-8 = 1 hr trypsin digestion at 37°C, 800ng trypsin (~1:10); samples 9-10 = 2 hr trypsin digestion at 37°C, 200ng trypsin; samples 11-12 = 2 hr trypsin digestion at 37°C, 600ng trypsin; samples 13-14 = 2 hr trypsin digestion at 37°C, 300ng trypsin + 300ng LysC; samples 15-16 = 2 hr trypsin digestion at 37°C, 800ng trypsin; samples 17-18 = Overnight trypsin digestion at 37°C, 200ng trypsin; samples 19-21 = 2 hr trypsin digestion at 37°C, 200ng trypsin (extra for Peptide Assay); and samples 22-24 = overnight trypsin digestion at 37°C, 200ng trypsin.

[00142] Step 4. The sample prep continued for trypsin elution by adding 1µg trypsin in 100µl 50 mM TEAB to each sample and incubated for 1 hour at 37°C in a thermomixer shaking at 800 rpm.

[00143] Step 5. After 1 HR incubation tubes were placed onto magnet to remove beads and 90µL of supernatant was removed and 2µL 25 ng/µL GFP and 25µl single pot reduction/alkylation solution were added to each reaction and incubated at 95°C for 5 mins.

[00144] Step 6. After Reduction/Alkylation added digestion enzymes as stated in step 3 and incubated at stated times and temperatures for digestion reactions.

[00145] Step 7. Acidified the samples by adding 4.5µL 10%TFA + 1µL 25% TFA to acidify samples (pH< 3). Centrifuged all samples at 15,000 x g for 2mins and then removed 112 µL of samples and transferred to clean 1.5 ml low protein binding tube. Dried down the samples for about 1hr in speed vac. Added 17µL of QC peptide Mix (80fmol) in 0.2% FA and 4% ACN to samples 1-18. Reconstitute samples 19-24 in 17µL 4% ACN and MS grade water. Stored all samples at -20°C before nanoLC-MS/MS analysis.

[00146] Results are shown in Figures 13-14. In Figure 13A, for peptide area: 1hr with Trypsin/LysC combination passed specs (<20%) for all targets. In Figure 13B, 0% missed cleavage peptides: All targets passed specs across all conditions except KRAS/NRAS at 1hr.

[00147] The results of targeted analysis are shown in Figures 14A-F. Figures 14A-B show no significant difference found with different amount of trypsin or trypsin/LysC combination. Figures 14C and D show no significant difference found with different amount of trypsin or trypsin/LysC combo except 2hr with 800ng trypsin. Figures 14E-F show better recovery of most peptides for CTNNB1 and IQGAP1 observed with more trypsin (600-800ngs) or trypsin/LysC combo (600ng).

Example 8: Optimization of Enzymatic IP elution

[00148] Experiments were performed to test trypsin elution from beads by varying enzyme amounts and elution times. A flowchart is shown in Figure 1.

Experimental Protocol:

[00149] Step 1. Materials used are same as listed in Table 1. Prepared biotinylated antibody mixture as shown in Table 2 above and mixed with 0.5mg each of HCT116 (IGF Stim:Unstim)/HEK293 lysates. Performed total of 27 IP reactions by adding appropriate amount of antibody mix in 1.5 mL low protein binding tube. Parafiled each tube and incubated overnight at 4°C mixing on a rotator. After incubation added streptavidin magnetic beads using ratio of 1:5 (Antibody:Beads, because 10µg Antibody was used 50µL beads were added per 1mL). Washed beads with 2X volume cold IP Lysis Buffer twice. Removed wash buffer then added back original volume of IP Lysis Buffer to each tube. Pooled all IPs together in a 50mL conical tube and aliquoted into 25 1mL tubes and then added washed beads to each IP. Mixed

antibody/antigen samples and rotated for 1 hour at room temperature. Washed 3 times with 500µL Wash Buffer A. Washed 2 times with 500µL Wash Buffer B.

[00150] Step 2. The following MS sample prep solutions were prepared: 50mM TEAB – Diluted 1M Trimethylammonium Bicarbonate (TEAB) (PN#90114) to 50mM by adding 0.5mL TEAB in 9.5mL MS grade water, pH 8.5; Single Pot Reduction Alkylation – Final 50 mM TEAB pH 8.5; 10 mM TCEP; 20 mM chloroacetamide (CLAA) (Thermo Fisher Scientific; PN# A39270); 0.2µg/µL Trypsin Stock – Dissolved 20µg Trypsin protease (PN#90057) in 100µL 0.1% Acetic acid; Various trypsin stocks – 1µg trypsin = 5µL 0.2µg/µL trypsin stock + 95µL 50 mM TEAB solution then add 10µL to sample 1-9; 2µg trypsin = 10µL 0.2µg/µL trypsin stock + 90µL 50 mM TEAB solution then add 10µL to sample 10-18; 0.5µg trypsin = 2.5µL 0.2µg/µL trypsin stock + 97.5µL 50 mM TEAB solution then add 10µL to sample 19-27; 0.2% Formic Acid (FA), 4% Acetonitrile (ACN) - Add 940µL MS grade water, 20µL 10%FA and 40µL of 100% ACN.

[00151] Step 3. Mass Spec Sample Prep Methods tested included. Samples 1-3 = 1µg trypsin digestion 1 hour at 37°C; samples 4-6 = 1µg trypsin digestion 30 min at 37°C; samples 7-9 = 1µg trypsin digestion 15 min at 37°C; samples 10-12 = 2µg trypsin digestion 1 hour at 37°C; samples 13-15 = 2µg trypsin digestion 30 min at 37°C; samples 16-18 = 2µg trypsin digestion 15 min at 37°C; samples 19-21 = 0.5µg trypsin digestion 1 hour at 37°C; samples 22-24 = 0.5µg trypsin digestion 30 min at 37°C; samples 25-27 = 2µg trypsin digestion 15 min at 37°C.

[00152] Step 4. The sample prep continued for trypsin elution by adding 0.5, 1, or 2µg trypsin in 100µL 50 mM TEAB to each sample and incubated for 15 min, 30 min, or 1 hour at 37°C in a thermomixer shaking at 800 rpm.

[00153] Step 5. After 1 hour incubation tubes were placed onto magnet to remove beads and 90µL of supernatant was removed and 2µL 25 ng/µL GFP and 25µL single pot reduction/alkylation solution were added to each reaction and incubated at 95°C for 5 mins.

[00154] Step 6. After Reduction/Alkylation 1µg trypsin was added in 50mM TEAB and incubated for 2 hours at 37°C shaking at 800 rpm.

[00155] Step 7. Acidified the samples by adding 2.5µL 25% TFA to acidify samples (pH< 3). Centrifuged all samples at 15,000 x g for 2mins and then removed 112 µL of samples and transferred to clean 1.5 ml low protein binding tube. Dried down the samples for about 1hr in speed vac. Added 20µL of QC peptide Mix (80fmol) in 0.2% FA and 4% ACN to samples 1-18.

Reconstitute samples 19-24 in 17µL 4% ACN and MS grade water. Stored all samples at -20°C before nanoLC-MS/MS analysis.

[00156] Results are shown in Figures 16A-C, 17A-F. All graphs are plotted as % Control (trypsin elution using 1ug of trypsin for 1hour) i.e. Peptide Peak Area Intensities (Figure 16) or PRM ratios (Figure 17) obtained for all the samples were plotted considering results obtained for samples eluted using 1ug trypsin for 1 hour as 100.

[00157] Elution with 1ug and 500ng passes for all times while 2ug fails for 15 and 30 mins for most of the targets.

[00158] MS grade trypsin showed better recovery of targets and low trypsin autolysis peaks in LC-MS analysis.

Example 9: Optimization of trypsin elution from bead—time, amount and grade of enzyme.

[00159] Experiments were performed designed to assess enzymatic elution from IP beads by varying time, trypsin amount, and trypsin grade.

[00160] Step 1. Materials used are same as listed in Table 1 with Low grade trypsin. Prepared biotinylated antibody mixture as shown in Table 2 above and mixed with 0.5mg each of HCT116 (IGF Stim:Unstim)/HEK293 lysates. Performed total of 63 IP reactions by adding appropriate amount of antibody mix in 1.5 mL low protein binding tube. Parafilm each tube and incubated overnight at 4°C mixing on a rotator. After incubation added streptavidin magnetic beads using ratio of 1:5 (Antibody:Beads, because 10µg Antibody was used 50µL beads were added per 1mL). Washed beads with 2X volume cold IP Lysis Buffer twice. Removed wash buffer then added back original volume of IP Lysis Buffer to each tube. Pooled all IPs together in a 50mL conical tube and aliquoted into 25 1mL tubes and then added washed beads to each IP. Mixed antibody/antigen samples and rotated for 1 hour at room temperature. Washed 3 times with 500µL Wash Buffer A. Washed 2 times with 500µL Wash Buffer B.

[00161] Step 2. The following MS sample prep solutions were prepared: 50mM TEAB – Diluted 1M Trimethylammonium Bicarbonate (TEAB) (PN#90114) to 50mM by adding 0.5mL TEAB in 9.5mL MS grade water, pH 8.5; Single Pot Reduction Alkylation – Final 50 mM TEAB pH 8.5; 10 mM TCEP; 20 mM chloroacetamide (CLAA) (Thermo Fisher Scientific; PN# A39270); 0.2µg/µL Trypsin Stock – Dissolved 20µg Trypsin protease (MS grade PN#90057; Low grade PN#1879820) in 100µL 0.1% Acetic acid; For both types of trypsin - 100ng = Added

0.5µl 0.2µg/µl solution + 99.5µl 50 mM TEAB per samples 1-9 MS grade and 28-36 low grade trypsin; 500ng = Added 2.5µl 0.2µg/µl solution + 97.5µl 50 mM TEAB per samples 10-18 MS grade and 37-45 low grade trypsin; 1µg = Added 5µl 0.2µg/µl solution + 95µl 50 mM TEAB per samples 19-27 MS grade and 46-54 low grade trypsin; 1µg = Added 10µl 0.2µg/µl solution + 90µl 50 mM TEAB per samples 55-63 low grade trypsin; trypsin digestion 60ng/µL = 90µL trypsin stock + 60µL 50mM TEAB solution then add 10µL to all samples; 0.2% Formic Acid (FA), 4% Acetonitrile (ACN) - Add 940µL MS grade water, 20µL 10%FA and 40µL of 100% ACN.

[00162] Step 3. Mass Spec Sample Prep Methods tested included. Samples 1-3 = 15 min trypsin digestion at 37°C, 100ng MS grade trypsin; samples 4-6 = 30 min trypsin digestion at 37°C, 100ng MS grade trypsin; samples 7-9 = 1-hour trypsin digestion at 37°C, 100ng MS grade trypsin; samples 10-12 = 15 min trypsin digestion at 37°C, 500ng MS grade trypsin; samples 13-15 = 30 min trypsin digestion at 37°C, 500ng MS grade trypsin; samples 16-18 = 1-hour trypsin digestion at 37°C, 500ng MS grade trypsin; samples 19-21 = 15 min trypsin digestion at 37°C, 1µg MS grade trypsin; samples 22-24 = 30 min trypsin digestion at 37°C, 1µg MS grade trypsin; samples 25-27 = 1-hour trypsin digestion at 37°C, 1µg MS grade trypsin; samples 28-30 = 15 min trypsin digestion at 37°C, 100ng low grade trypsin; samples 31-33 = 30 min trypsin digestion at 37°C, 100ng low grade trypsin; samples 34-36 = 1-hour trypsin digestion at 37°C, 100ng low grade trypsin; samples 37-39 = 15 min trypsin digestion at 37°C, 500ng low grade trypsin; samples 40-42 = 30 min trypsin digestion at 37°C, 500ng low grade trypsin; samples 43-45 = 1-hour trypsin digestion at 37°C, 500ng low grade trypsin; samples 46-48 = 15 min trypsin digestion at 37°C, 1µg low grade trypsin; samples 49-51 = 30 min trypsin digestion at 37°C, 1µg low grade trypsin; samples 52-54 = 1-hour trypsin digestion at 37°C, 1µg low grade trypsin; samples 55-57 = 15 min trypsin digestion at 37°C, 2µg low grade trypsin; samples 58-60 = 30 min trypsin digestion at 37°C, 2µg low grade trypsin; samples 61-63 = 1-hour trypsin digestion at 37°C, 2µg low grade trypsin;

[00163] Step 4. The sample prep continued for trypsin elution by adding stated amounts and type of trypsin in 100µl 50 mM TEAB to each sample and incubated for stated times at 37°C in a thermomixer shaking at 500 rpm.

[00164] Step 5. After 1 HR incubation tubes were placed onto magnet to remove beads and 90 μ L of supernatant was removed and 2 μ L 25 ng/ μ L GFP and 25 μ L single pot reduction/alkylation solution were added to each reaction and incubated at 95°C for 5 mins.

[00165] Step 6. After Reduction/Alkylation added 60 ng/ μ L digestion trypsin was added and incubated at 37°C for 2 hours shaking at 500 rpm.

[00166] Step 7. Acidified the samples by adding 2.5 μ L 25% TFA to acidify samples (pH< 3). Centrifuged all samples at 15,000 x g for 2mins and then removed 112 μ L of samples and transferred to clean 1.5 ml low protein binding tube. Dried down the samples for about 1hr in speed vac. Added 17 μ L of QC peptide Mix (80fmol) in 0.2% FA and 4% ACN to samples 1-18. Reconstitute samples 19-24 in 17 μ L 4% ACN and MS grade water. Stored all samples at -20°C before nanoLC-MS/MS analysis.

[00167] Results are shown in Figures 19A-B. Graphs are plotted as % Control (Samples eluted using 1 μ g MS grade trypsin for 1hour).

[00168] Elution using 500ng Low grade or MS grade trypsin at all time points showed drop in intensities for most of the targets.

[00169] 1 μ g Low grade – drop in intensities, 2 failed at 30mins, 1 at 1 hour.

[00170] 1 μ g MS grade – equivalent or better intensities for 15 and 30 mins as compared to elution at 1 hour.

[00171] 2 μ g Low grade – all targets pass, slightly lower intensities as compared to control.

[00172] KRAS with low grade trypsin showed 400-500% increase compared to control.

[00173] Figure 20A-B provides tables showing % CVs for Example 8 above (Figure20A) and this Example 9 (Figure 20B).

[00174] Figure 21 provides a comparison of results for 1 μ g trypsin elution from Examples 8 and 9.

Example 10: Certain Embodiments

[00175] The following numbered items provide additional support for and descriptions of the embodiments herein.

[00176] Item 1. A method for detecting one or more target protein(s) in a biological sample, comprising

- a. enriching the target protein(s) from a biological sample by binding the target protein(s) to a solid support;

- b. fragmenting the enriched target protein(s) by:
 - i. while bound to the solid support, treating the enriched target protein(s) with a first enzymatic digestion,
 - ii. reducing and alkylating the digested target protein(s) in a single reaction vessel, and
 - iii. digesting the reduced, alkylated, and digested target protein(s) in a second enzymatic digestion, wherein optionally the second enzymatic digestion is allowed to proceed for up to 18 hours (for example up to 4 hours); and
- c. detecting one or more target protein(s) in the sample.

[00177] Item 2. The method of item 1, wherein the solid support comprises a bead or a resin.

[00178] Item 3. The method of item 1, wherein the solid support comprises a magnetic bead.

[00179] Item 4. The method of item 1, wherein the solid support comprises an immunoaffinity bead.

[00180] Item 5. The method of item 1, wherein enriching the target protein(s) from a biological sample by binding the target protein(s) to a solid support comprises treating the biological sample with at least one antibody capable of immunoaffinity enriching the target protein(s) from a biological sample.

[00181] Item 6. The method of any one of items 1-5, wherein detecting one or more target proteins(s) in the sample comprises assaying the fragmented protein(s) via mass spectrometry to determine the presence or absence of at least one peptide from the target protein(s).

[00182] Item 7. The method of item 6, wherein the peptide is less than or equal to 40 amino acids in length.

[00183] Item 8. The method of any one of items 1-7, wherein detecting one or more target protein(s) in the sample comprises ELISA, Western blot, bead-based multianalyte profiling (such as Luminex), fluorescence-based imaging, or chemiluminescent-based imaging.

- [00184] Item 9. The method of any one of items 1-8, wherein the first and/or second enzymatic digestion comprises digestion with trypsin, chymotrypsin, AspN, GluC, LysC, LysN, ArgC, proteinase K, pepsin, clostripain, elastase, GluC biocarb, LysC/P, LysN promisc, protein endopeptidase, staph protease or thermolysin.
- [00185] Item 10. The method of item 9, wherein the first and/or second enzymatic digestion comprises digestion with trypsin.
- [00186] Item 11. The method of item 9, wherein the first and/or second enzymatic digestion comprises digestion with trypsin and LysC.
- [00187] Item 12. The method of any one of items 9-11, wherein the trypsin is present in the first enzymatic digestion at an amount of 0.5 µg to 2 µg or an amount of 0.1 µg/µl to 0.4 µg/µl.
- [00188] Item 13. The method of any one of items 9-11, wherein the trypsin is present in the second enzymatic digestion at an amount of 0.2 µg to 0.8 µg or an amount of 0.02 µg/µl to 0.08 µg/µl.
- [00189] Item 14. The method of any one of items 1-13, wherein the reduction/alkylation step comprises mixing the product of the first enzymatic digestion with a solution comprising TCEP and chloroacetamide.
- [00190] Item 15. The method of item 14, wherein the TCEP and chloroacetamide are present in a ratio of 1:1, 1:2, 1:3, 1:4, or 1:5.
- [00191] Item 16. The method of items any one of 1-15, further comprising the step of neutralization after the second digestion and prior to mass spectrometry.
- [00192] Item 17. The method of item 16, wherein the neutralization step comprises adding trifluoroacetic acid (TFA) to the product of the second enzymatic digestion.
- [00193] Item 18. The method of any one of items 1-17, wherein step a) comprises treating the sample with a labelled antibody capable of binding to the target protein to provide a labelled antibody-protein conjugate; and binding the labelled antibody-protein conjugate with a capture agent capable of binding to the labelled antibody to isolate the target protein from the sample.
- [00194] Item 19. The method of item 17, wherein the label is biotin and the capture agent is streptavidin.
- [00195] Item 20. The method of items any one of 1-19, wherein the lower limit of detection for the protein(s) is from 0.04 to 11.11 fmol.

- [00196] Item 21. The method of any one of items 1-20, further comprising determining the quantity of the target protein.
- [00197] Item 22. The method of item 21, wherein the quantity of a target protein is determined by adding an internal standard peptide of known amount to the digested protein prior to mass spectrometry, wherein the internal standard peptide has the same amino acid sequence as a target peptide, and is detectably labeled, and determining the quantity of a target peptide by comparison to the internal standard.
- [00198] Item 23. The method of item 21, wherein the quantity of a target protein is determined by a method comprising comparing an amount of a target peptide in the sample to the amount of the same target peptide in a control sample.
- [00199] Item 24. The method of any one of items 21 to 23, further comprising quantifying the relative amount of the target protein.
- [00200] Item 25. The method of any one of items 21 to 24, further comprising quantifying the absolute amount of the target protein.
- [00201] Item 26. The method of any one of items 21 to 25, wherein the lower limit of quantification is from 0.04 to 11.11 fmol.
- [00202] Item 27. The method of any one of items 1-26, further comprising desalting after fragmentation and prior to mass spectrometry.
- [00203] Item 28. The method of any one of items 1-27, wherein the mass spectrometry is selected from targeted mass spectrometry and discovery mass spectrometry.
- [00204] Item 29. The method of item 21, wherein the targeted mass spectrometry is selected from multiple reaction monitoring (MRM), selected reaction monitoring (SRM), and parallel reaction monitoring (PRM), or combinations thereof.
- [00205] Item 30. The method of any one of items 1-29, wherein the biological sample is selected from isolated cells, plasma, serum, whole blood, CSF, urine, sputum, tissue, and tumorous tissue.
- [00206] Item 31. The method of any one of items 1-30, wherein the biological sample is human.
- [00207] Item 32. The method of any one of items 1-31, wherein the peptide from the target protein(s) comprises an epitope for the antibody capable of immunoaffinity enriching the target protein(s).

[00208] Item 33. The method of any one of items 1-32, wherein the digestion is complete in 4 hours or less.

[00209] Item 34. The method of any one of items 1-33, wherein the method further comprises separating the solid support from digested protein(s).

[00210] Item 35. The method of any one of items 1-34, wherein the second enzymatic digestion is allowed to proceed for up to 18 hours.

[00211] Item 35. The method of claim 35, wherein the second enzymatic digestion is allowed to proceed for up to 4 hours.

We claim:

1. A method for detecting one or more target protein(s) in a biological sample, comprising
 - a. enriching the target protein(s) from a biological sample by binding the target protein(s) to a solid support;
 - b. fragmenting the enriched target protein(s) by:
 - i. while bound to the solid support, treating the enriched target protein(s) with a first enzymatic digestion,
 - ii. reducing and alkylating the digested target protein(s) in a single reaction vessel, and
 - iii. digesting the reduced, alkylated, and digested target protein(s) in a second enzymatic digestion, wherein optionally the second enzymatic digestion is allowed to proceed for up to 18 hours;
 - c. detecting one or more target protein(s) in the sample.
2. The method of claim 1, wherein enriching the target protein(s) from a biological sample by binding the target protein(s) to a solid support comprises treating the biological sample with at least one antibody capable of immunoaffinity enriching the target protein(s) from a biological sample.
3. The method of claim 1, wherein detecting one or more target proteins(s) in the sample comprises assaying the fragmented protein(s) via mass spectrometry to determine the presence or absence of at least one peptide from the target protein(s).
4. The method of claim 3, wherein the peptide is less than or equal to 40 amino acids in length.
5. The method of claims 1, wherein detecting one or more target protein(s) in the sample comprises ELISA, Western blot, bead-based multianalyte profiling, fluorescence-based imaging, or chemiluminescent-based imaging.
6. The method of claim 1, wherein the first and/or second enzymatic digestion comprises digestion with trypsin, chymotrypsin, AspN, GluC, LysC, LysN, ArgC, proteinase K,

pepsin, clostripain, elastase, GluC biocarb, LysC/P, LysN promisc, protein endopeptidase, staph protease or thermolysin.

7. The method of claim 1, wherein the first and/or second enzymatic digestion comprises digestion with trypsin.
8. The method of claim 1, wherein the first and/or second enzymatic digestion comprises digestion with trypsin and LysC.
9. The method of claim 1, wherein the reduction/alkylation step comprises mixing the product of the first enzymatic digestion with a solution comprising TCEP and chloroacetamide.
10. The method of claim 9, wherein the TCEP and chloroacetamide are present in a ratio of 1:1, 1:2, 1:3, 1:4, or 1:5.
11. The method of claim 1, further comprising the step of neutralization after the second digestion and prior to mass spectrometry.
12. The method of claim 11, wherein the neutralization step comprises adding trifluoroacetic acid (TFA) to the product of the second enzymatic digestion.
13. The method of claim 1, wherein step a) comprises treating the sample with a labelled antibody capable of binding to the target protein to provide a labelled antibody-protein conjugate; and binding the labelled antibody-protein conjugate with a capture agent capable of binding to the labelled antibody to isolate the target protein from the sample.
14. The method of claim 13, wherein the label is biotin and the capture agent is streptavidin.
15. The method of claim 1, wherein the lower limit of detection for the protein(s) is from 0.04 to 11.11 fmol.
16. The method of claim 1, further comprising determining the quantity of the target protein by adding an internal standard peptide of known amount to the digested protein prior to mass spectrometry, wherein the internal standard peptide has the same amino acid sequence as a target peptide, and is detectably labeled, and determining the quantity of a target peptide by comparison to the internal standard.
17. The method of claim 16, wherein the quantity of a target protein is determined by a method comprising comparing an amount of a target peptide in the sample to the amount of the same target peptide in a control sample.

18. The method of claim 1, wherein the peptide from the target protein(s) comprises an epitope for the antibody capable of immunoaffinity enriching the target protein(s).
19. The method of claim 1, wherein the digestion is complete in 4 hours or less.
The method of claim 1, wherein the method further comprises separating the solid support from digested protein(s).

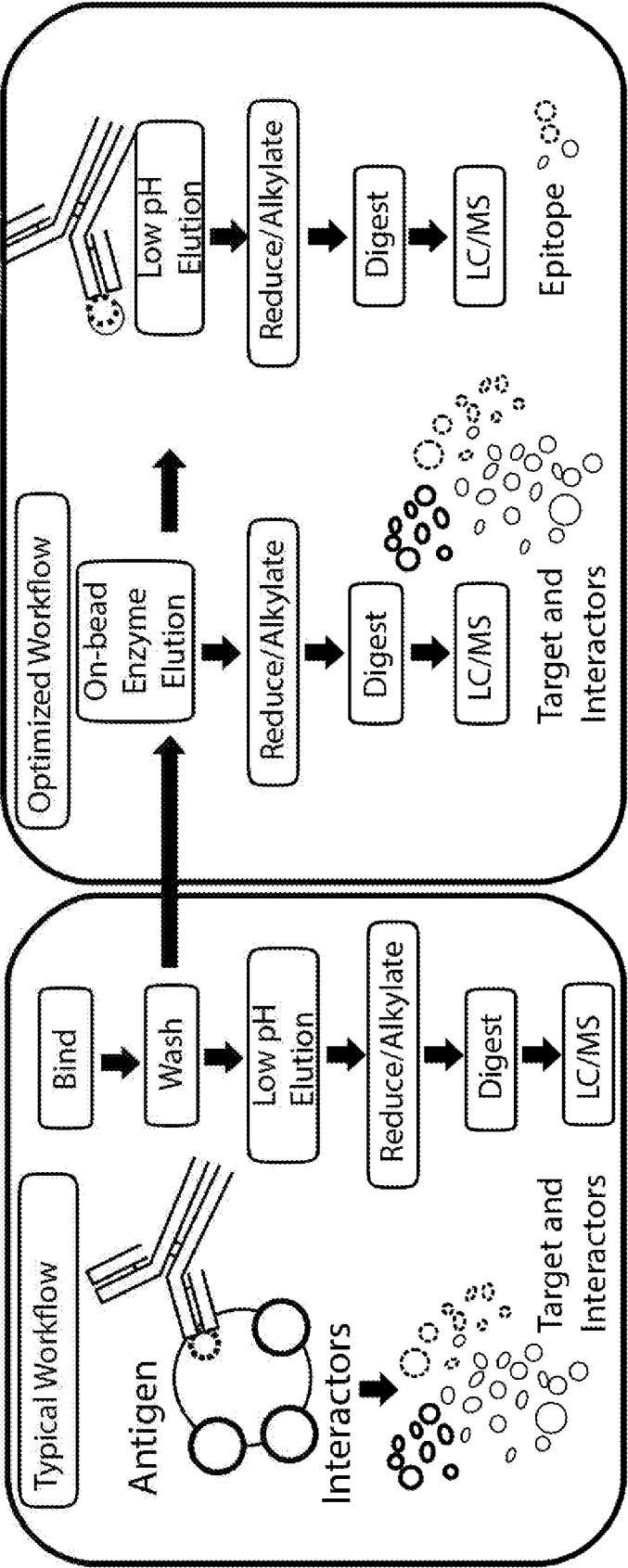


Figure 1

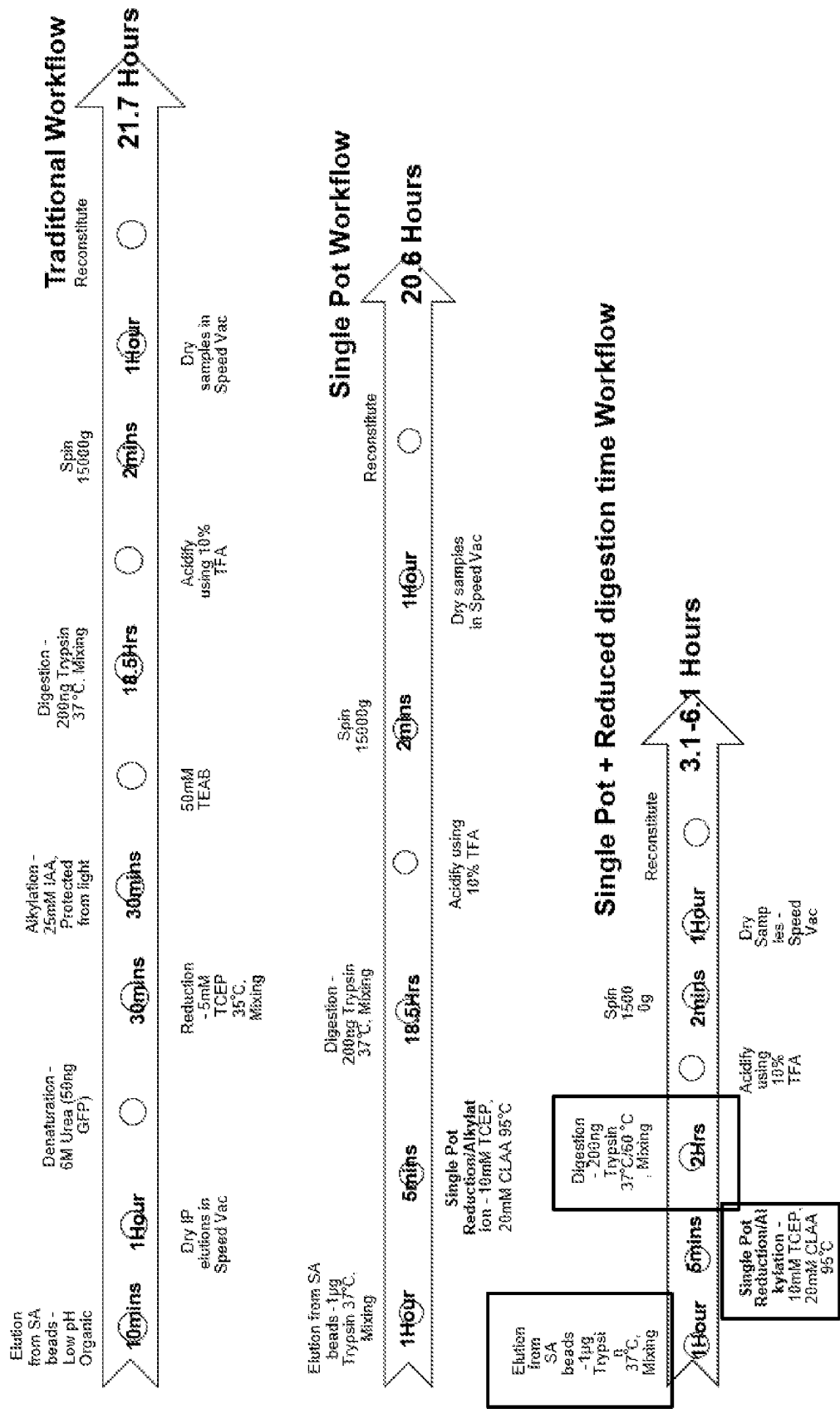


Figure 2

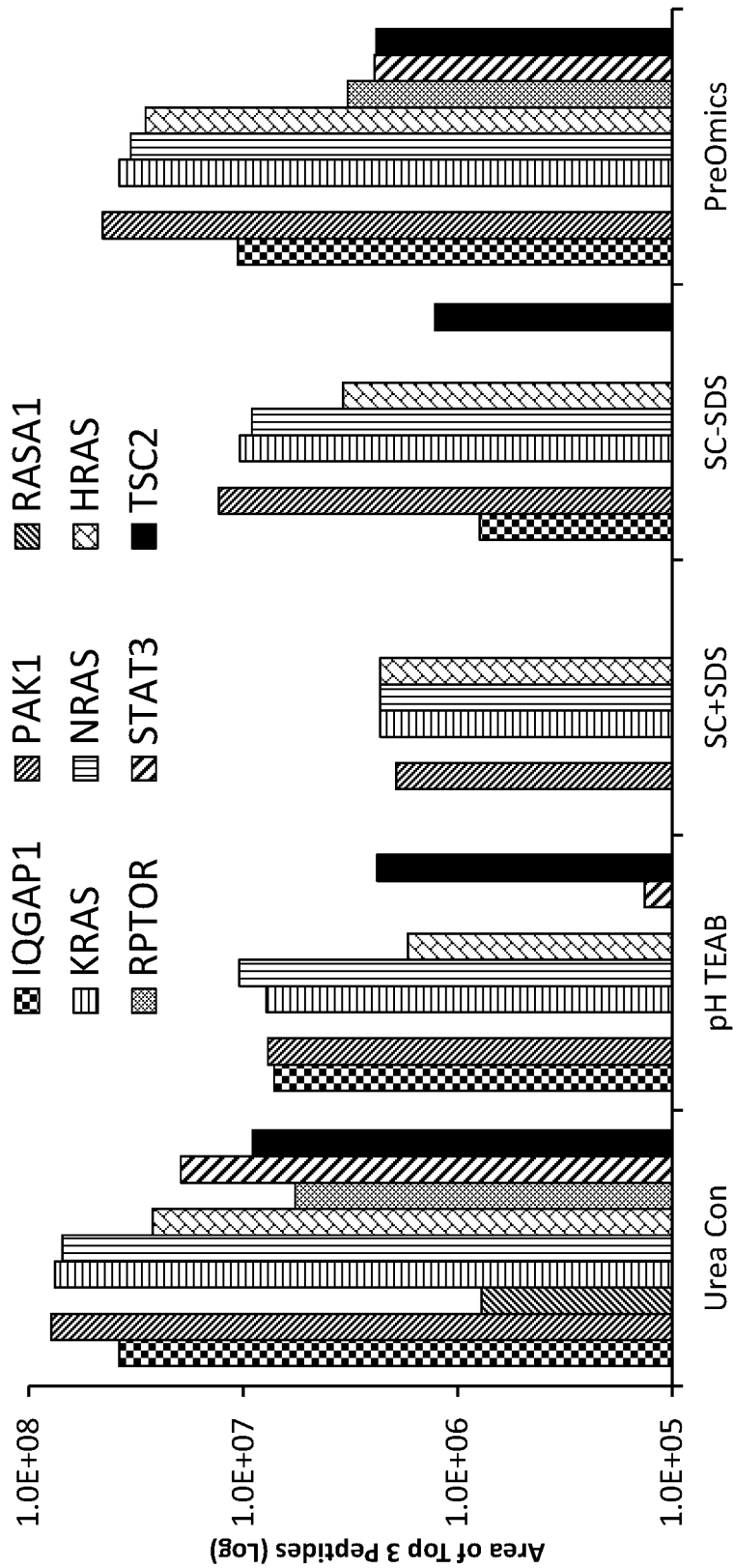


Figure 3

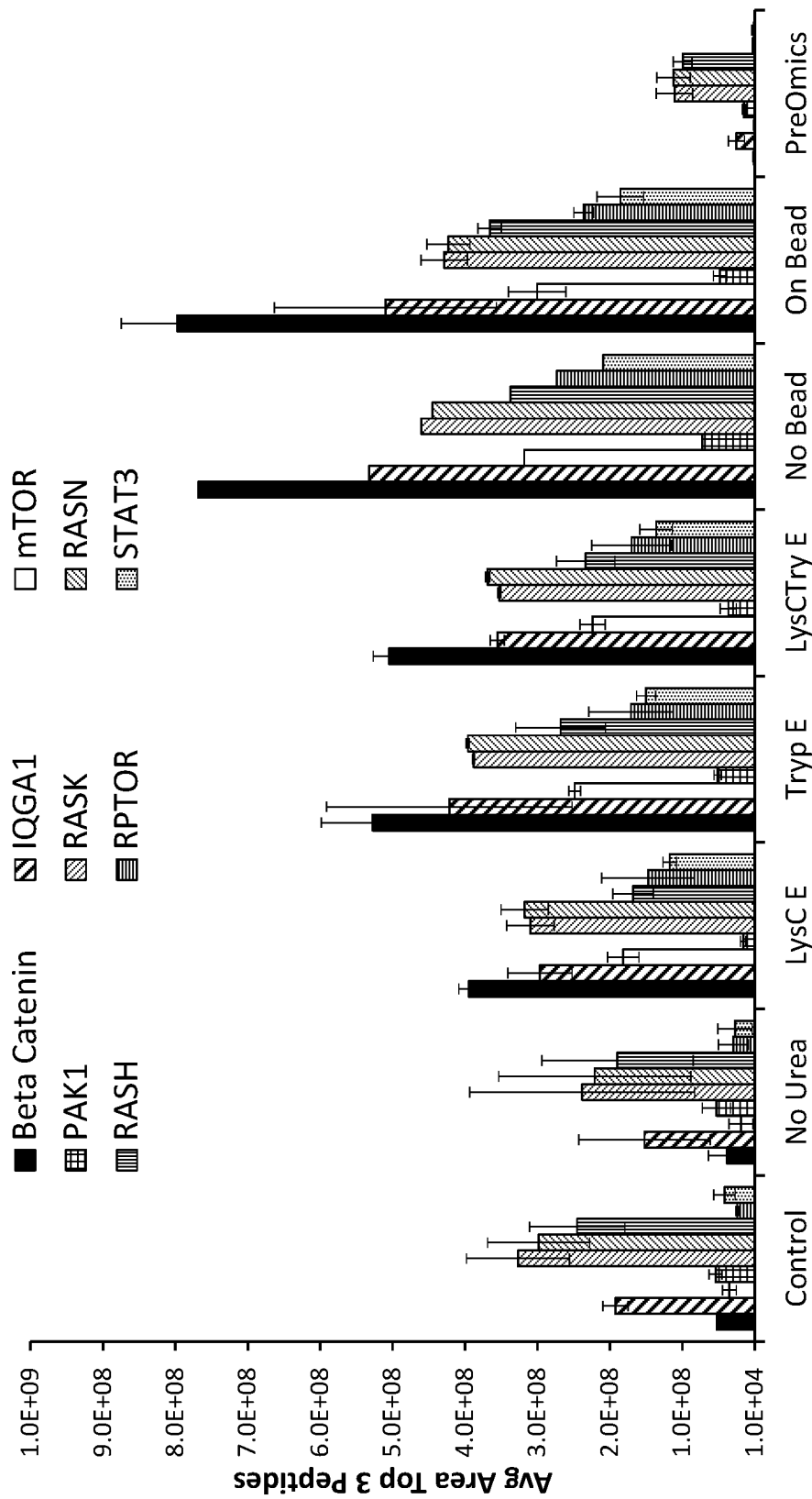


Figure 4

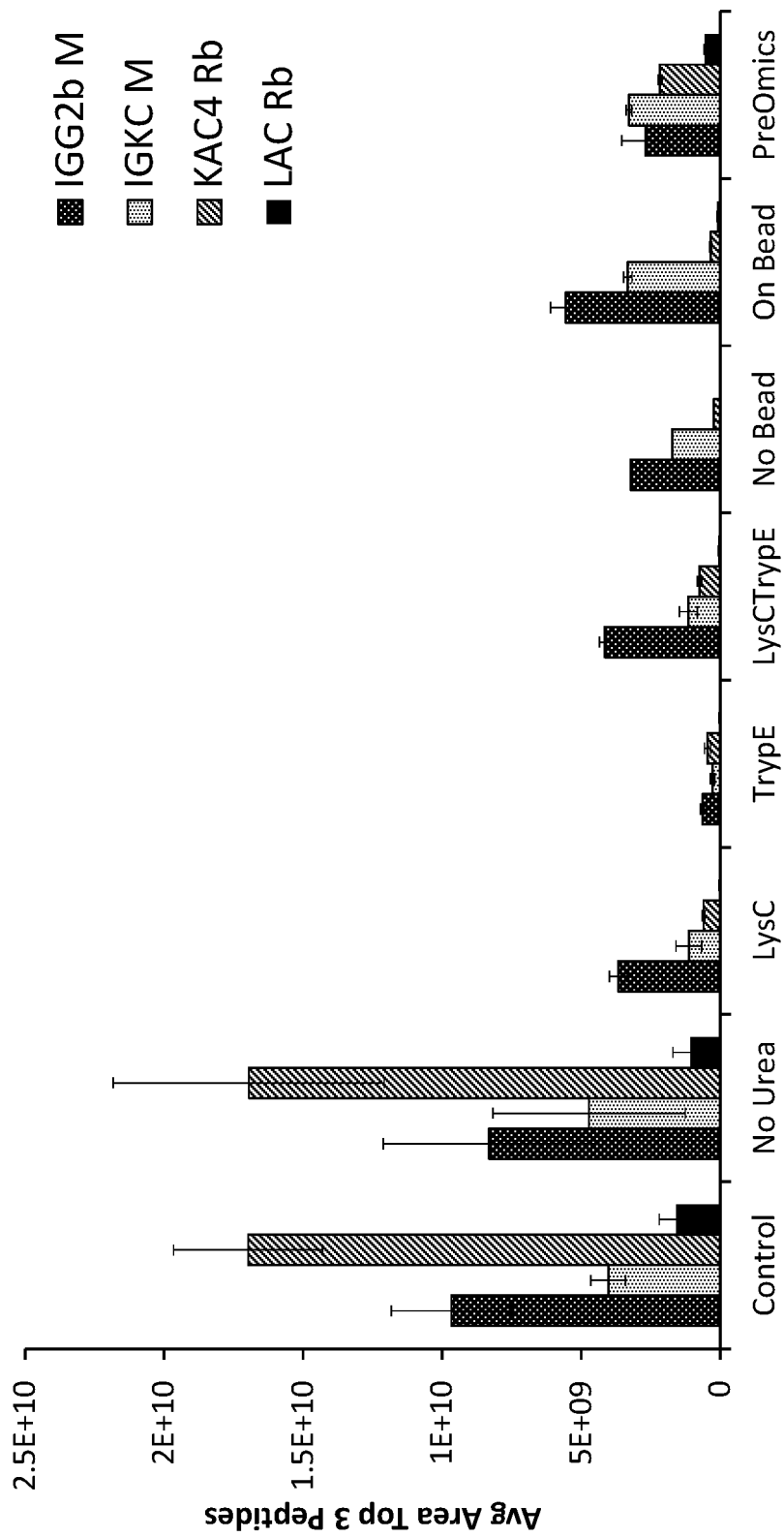


Figure 5

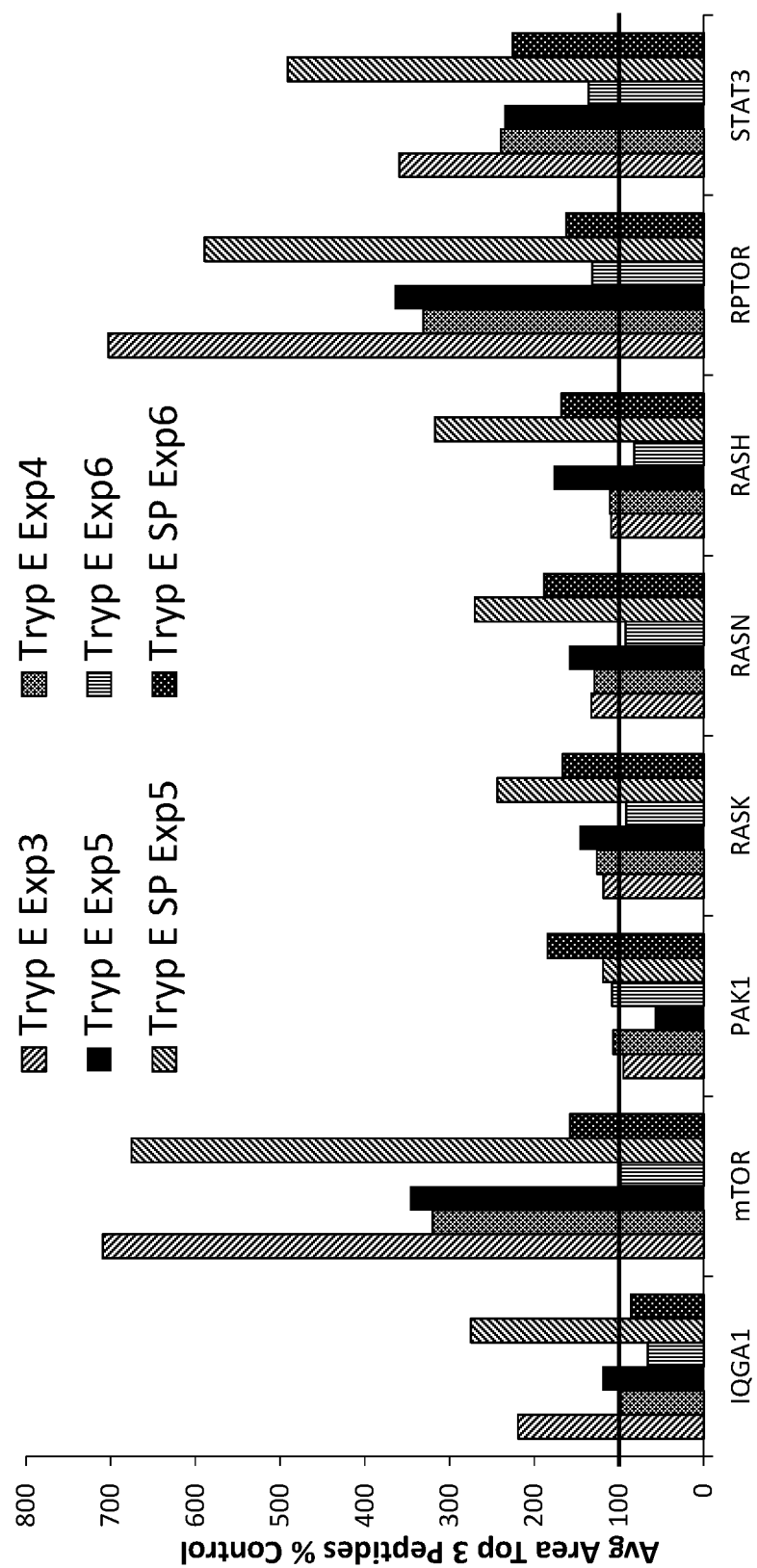


Figure 6

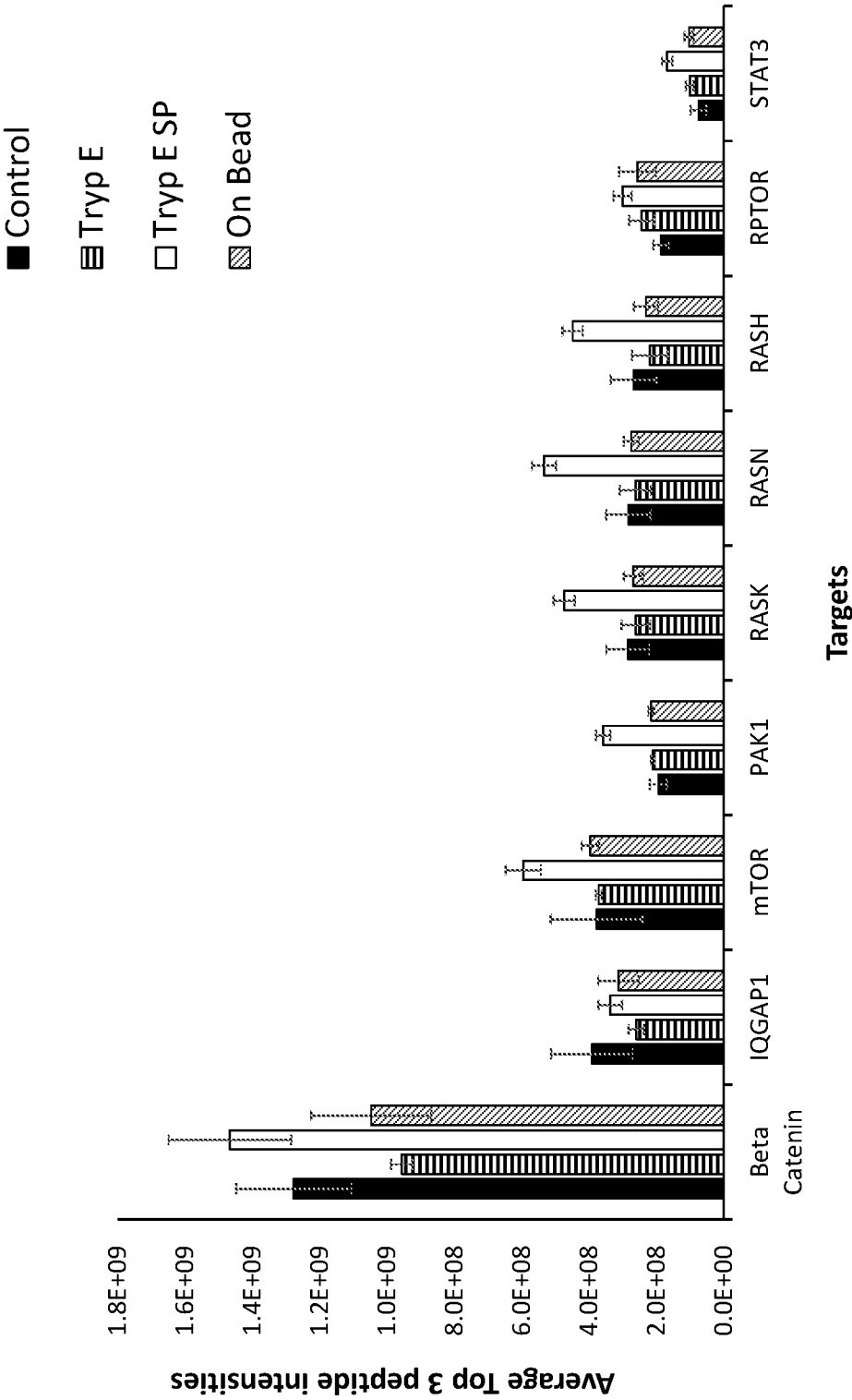


Figure 7

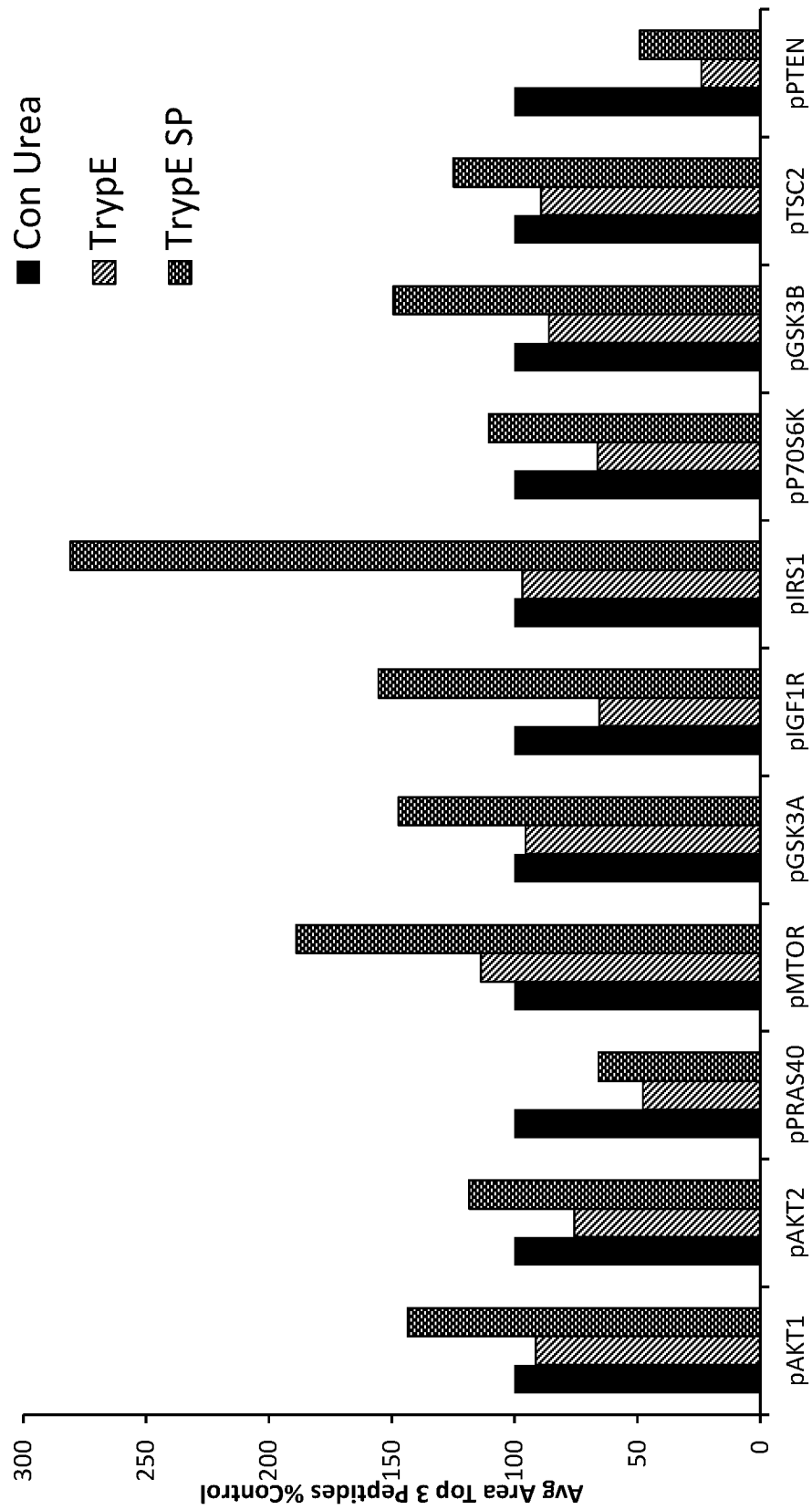


Figure 8

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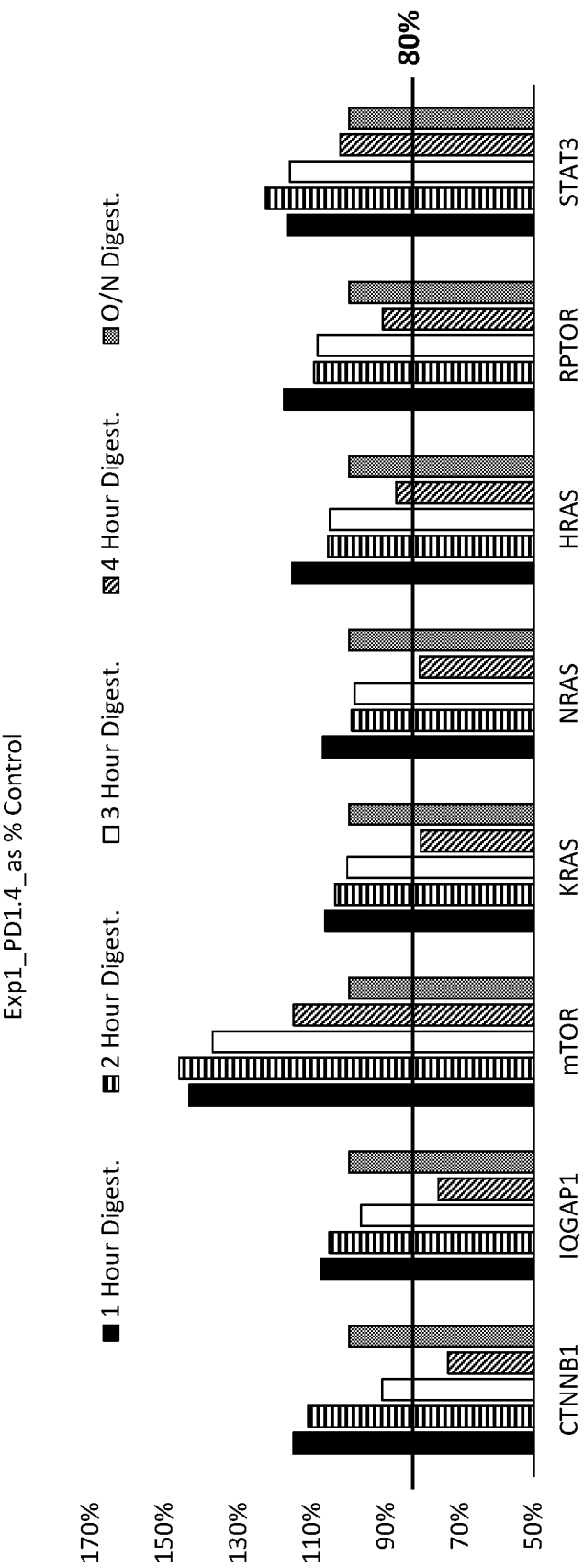


Figure 9A

10/31

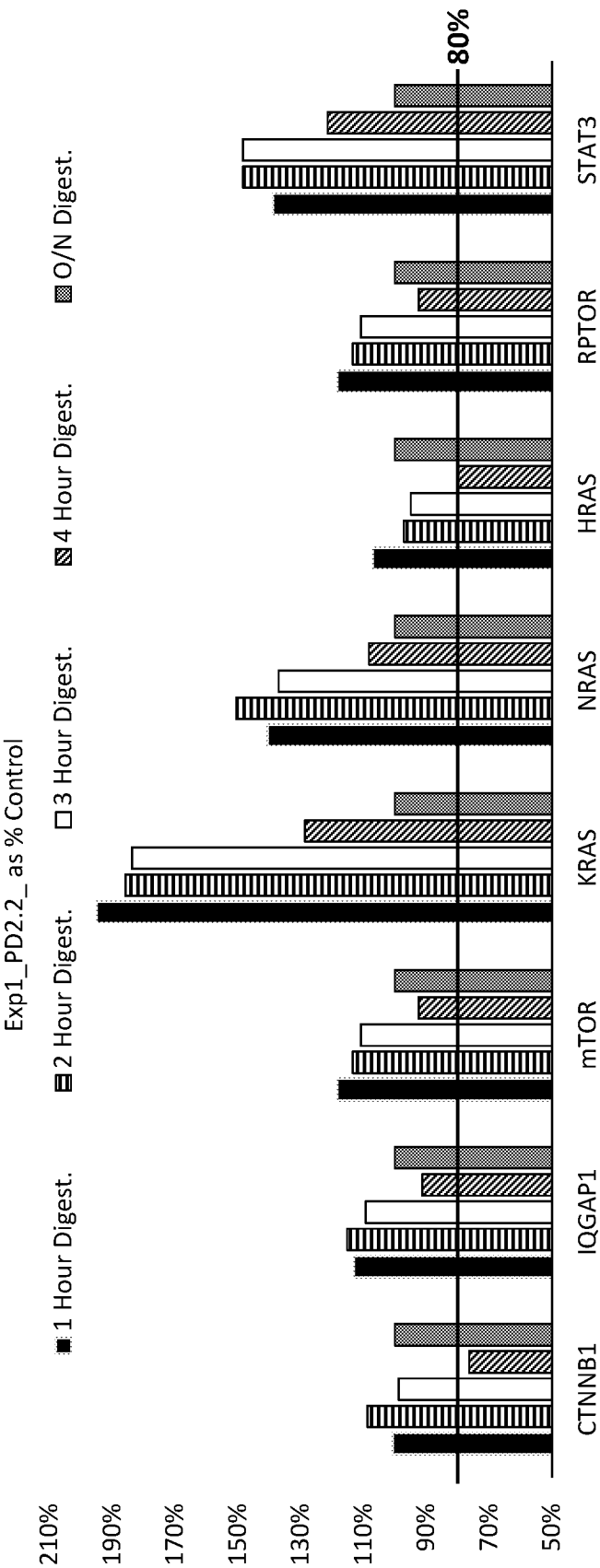


Figure 9B

11/31

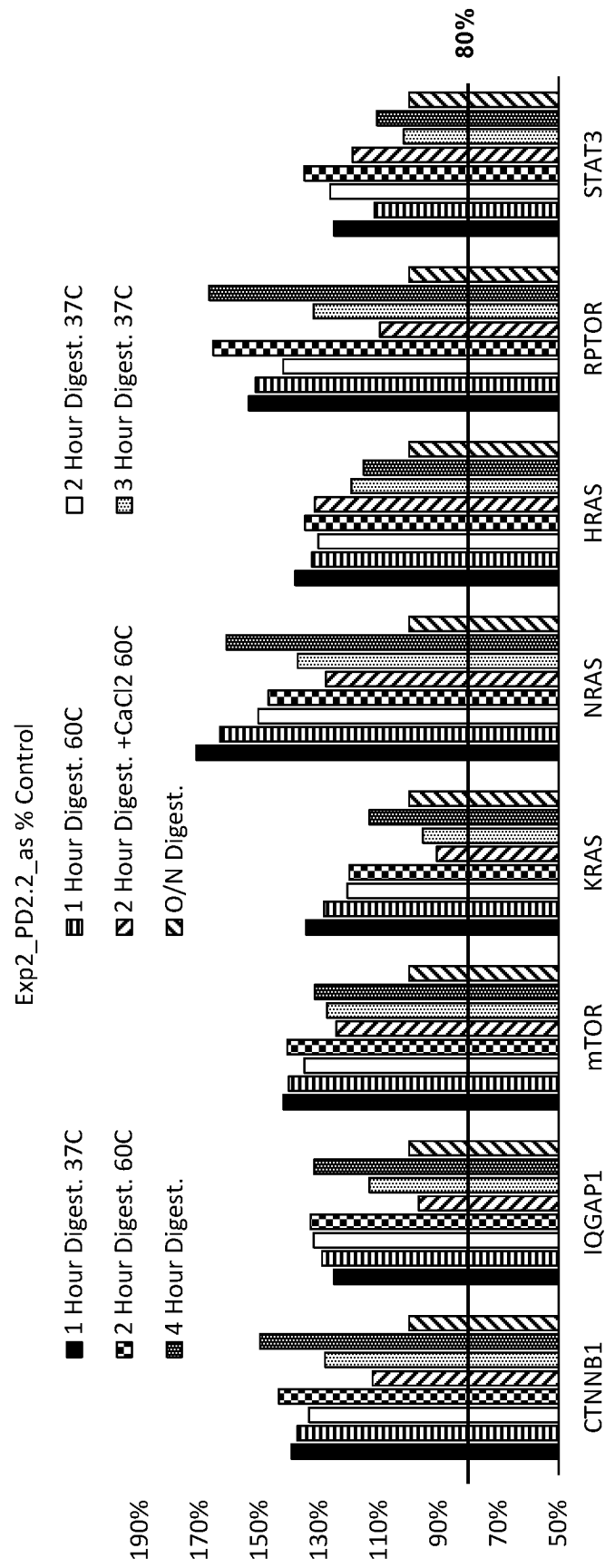


Figure 10A

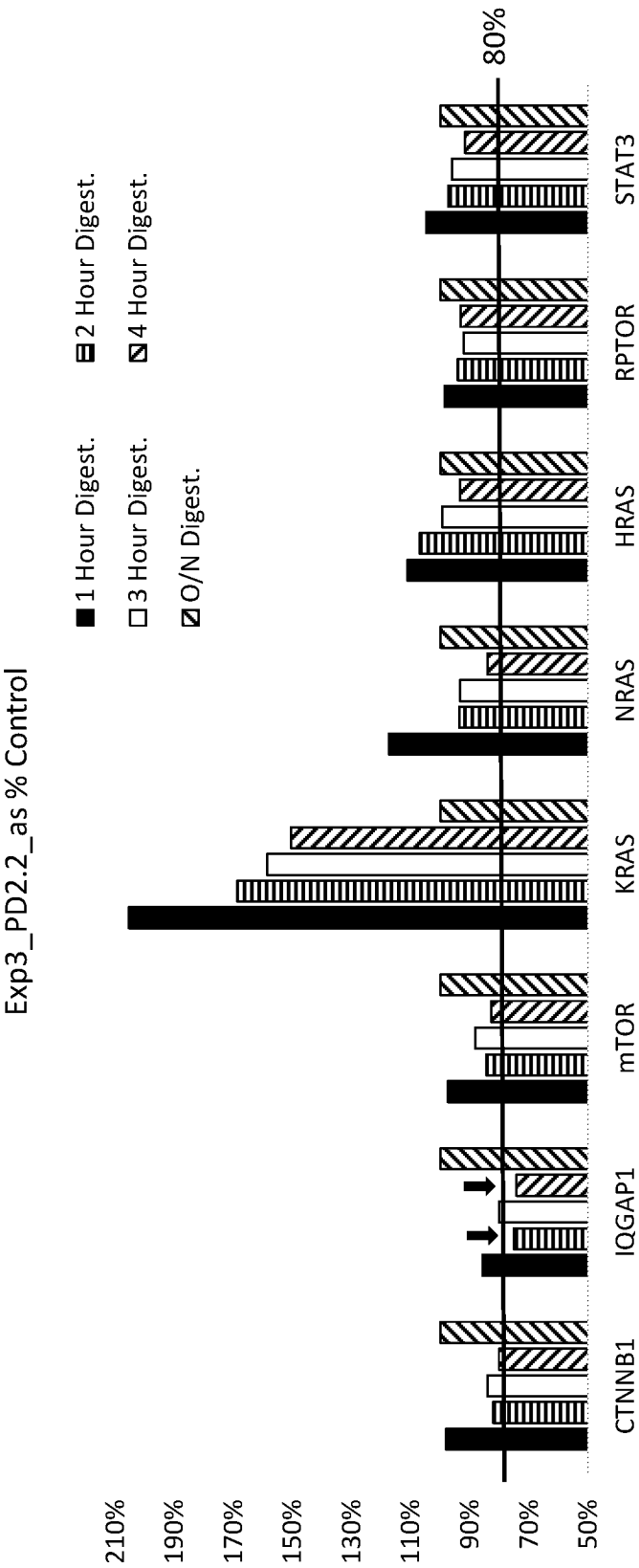


Figure 10B

Exp1

%CV	1 Hour Digest.	2 Hour Digest.	3 Hour Digest.	4 Hour Digest.	O/N Digest.
CTNNB1	18%	24%	8%	8%	0%
IQGAP1	14%	22%	10%	3%	10%
mTOR	14%	10%	4%	8%	4%
KRAS	17%	9%	12%	1%	10%
NRAS	19%	33%	22%	5%	7%
HRAS	11%	5%	1%	10%	6%
RPTOR	14%	10%	4%	8%	4%
STAT3	14%	9%	4%	3%	5%

Figure 11A

Exp2

%CV	1 Hour Digest. 37C	1 Hour Digest. 60C	2 Hour Digest. 37C	2 Hour Digest. 60C	2 Hour Digest. +CaCl ₂ 60C	3 Hour Digest. 37C	4 Hour Digest.	O/N Digest.
CTNNB1	14%	2%	7%	17%	26%	2%	11%	35%
IQGAP1	27%	15%	15%	39%	38%	13%	21%	18%
mTOR	3%	4%	0%	15%	24%	1%	52%	8%
KRAS	3%	3%	2%	17%	16%	6%	17%	8%
NRAS	1%	6%	3%	10%	16%	7%	13%	9%
HRAS	1%	3%	6%	18%	18%	3%	17%	9%
RPTOR	15%	6%	1%	24%	15%	4%	6%	17%
STAT3	11%	4%	0%	23%	21%	0%	18%	18%

Figure 11B

Exp3

%CV	1 Hour Digest.	2 Hour Digest.	3 Hour Digest.	4 Hour Digest.	O/N Digest.
CTNNB1	5%	5%	15%	1%	27%
IQGAP1	4%	5%	10%	1%	20%
mTOR	7%	7%	19%	7%	28%
KRAS	5%	8%	15%	5%	25%
NRAS	5%	6%	25%	5%	28%
HRAS	4%	5%	10%	1%	20%
RPTOR	7%	9%	18%	5%	28%
STAT3	5%	5%	10%	3%	19%

Figure 11C

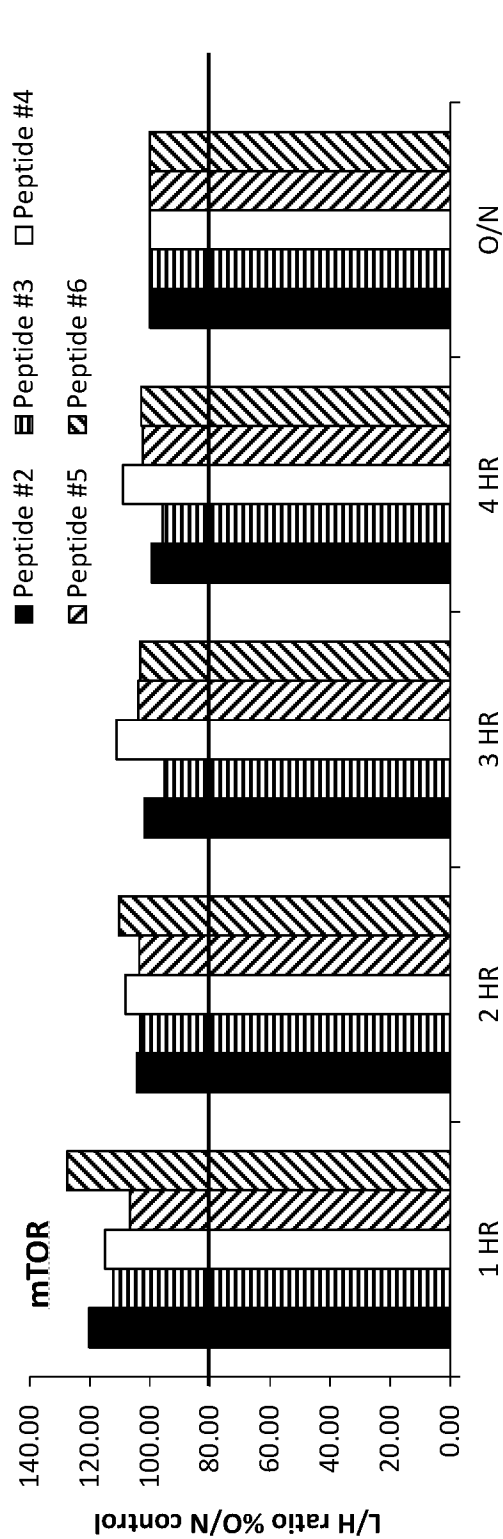


Figure 12A

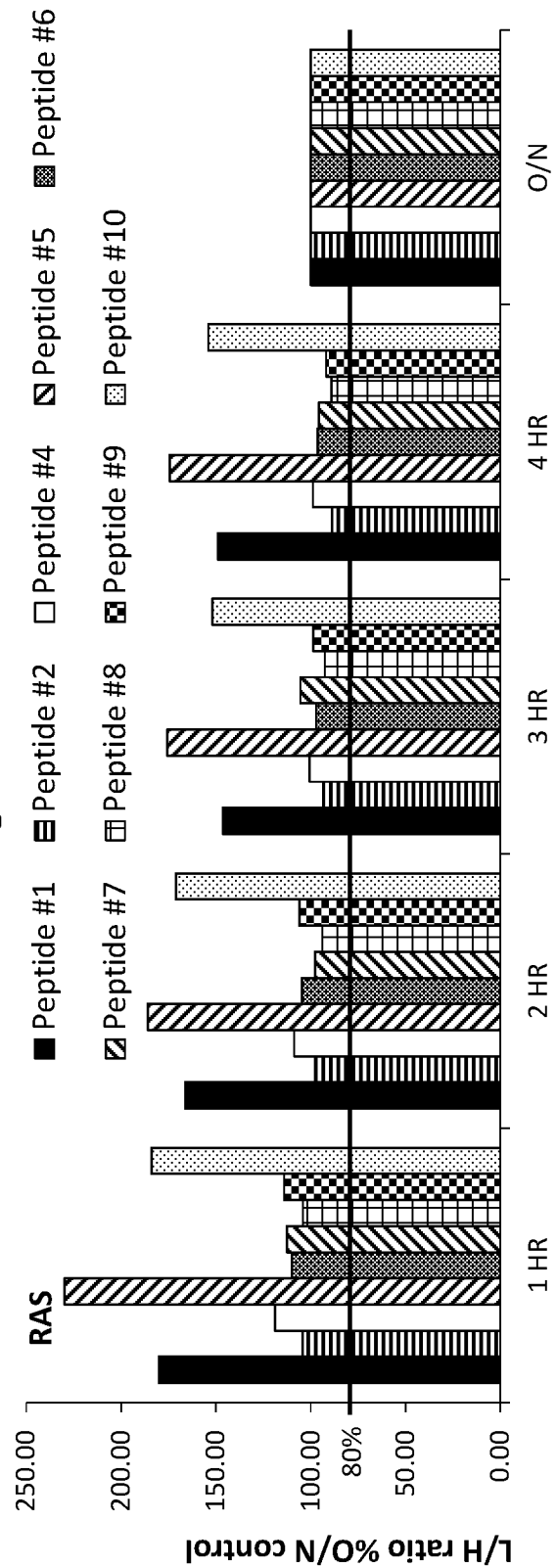


Figure 12B

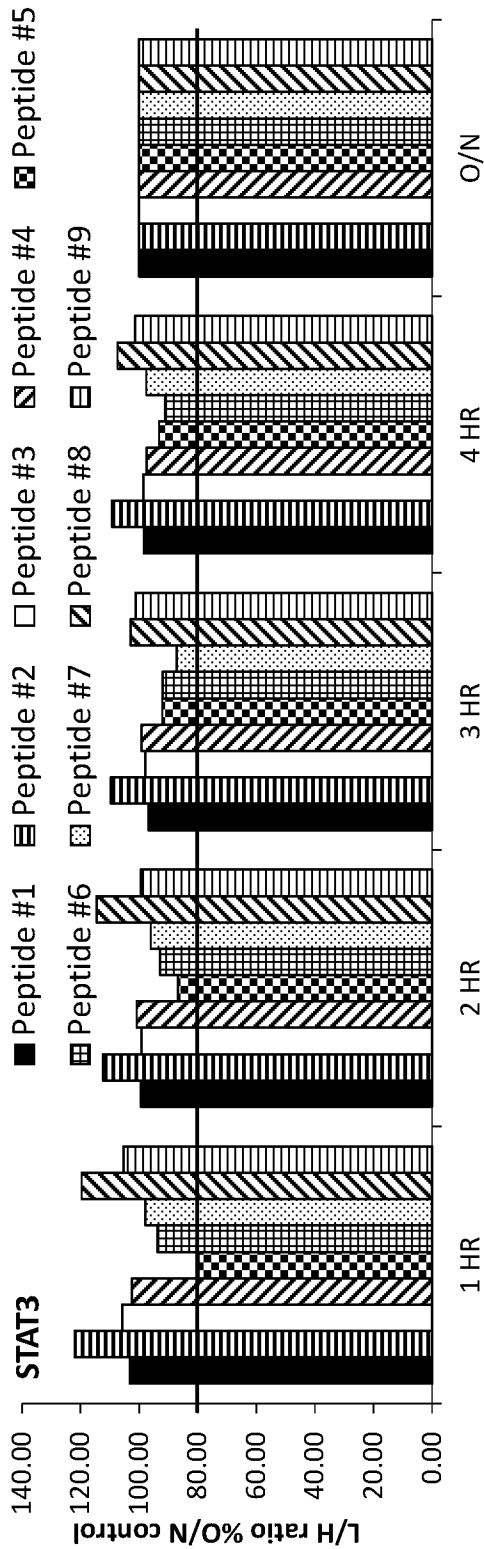


Figure 12C

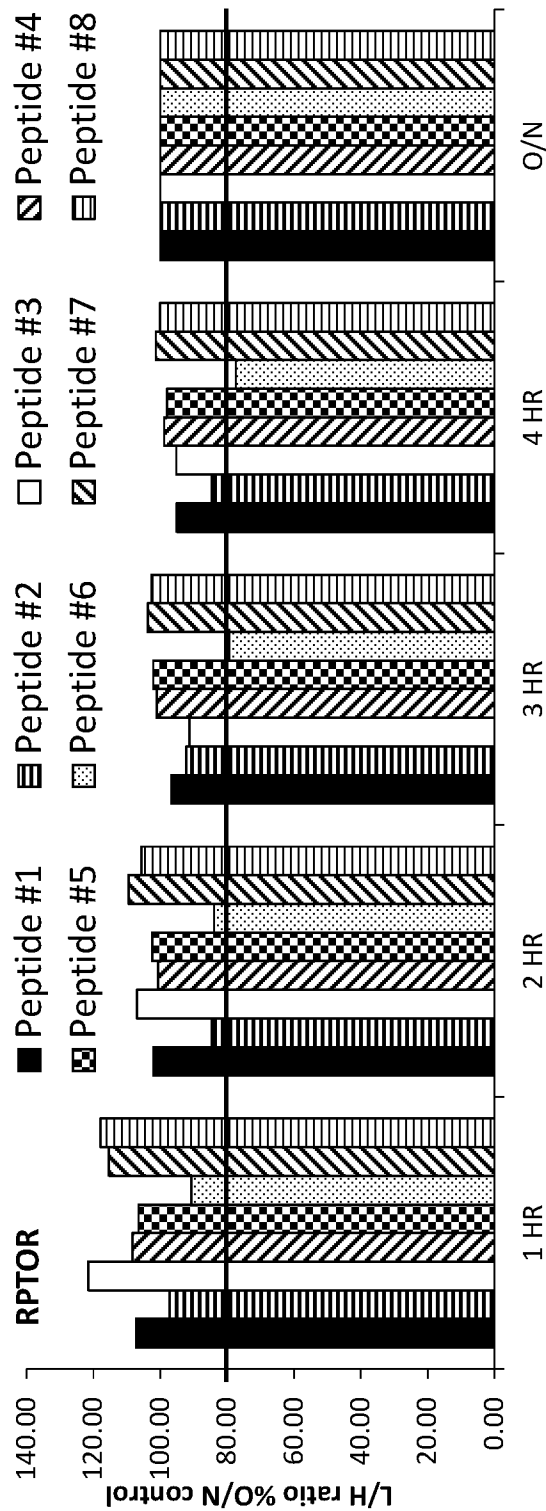


Figure 12D

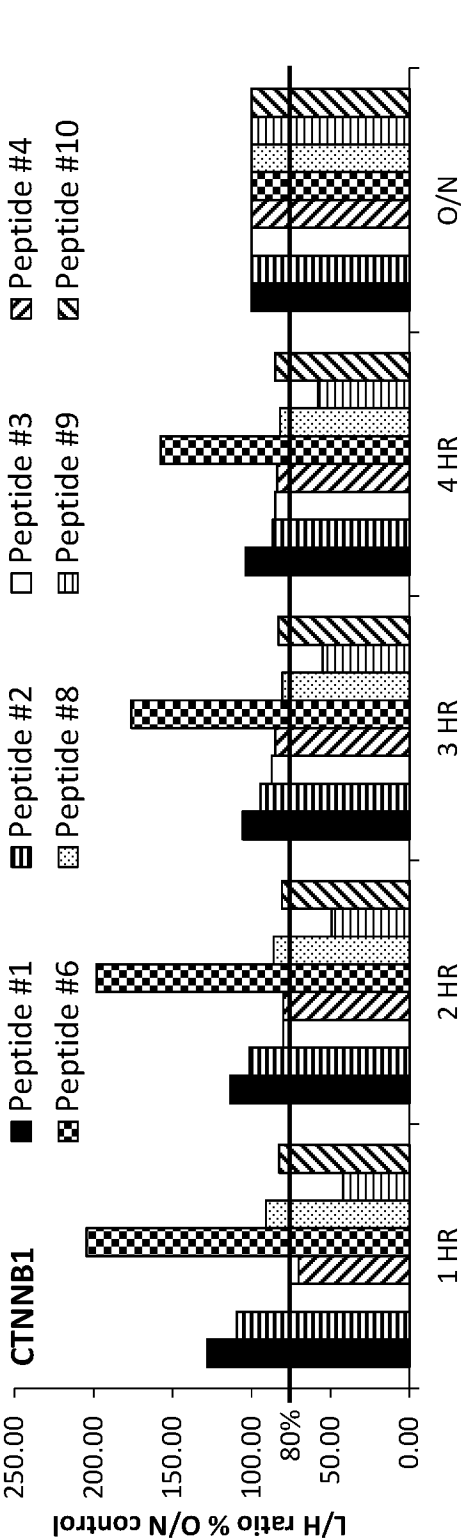


Figure 12E

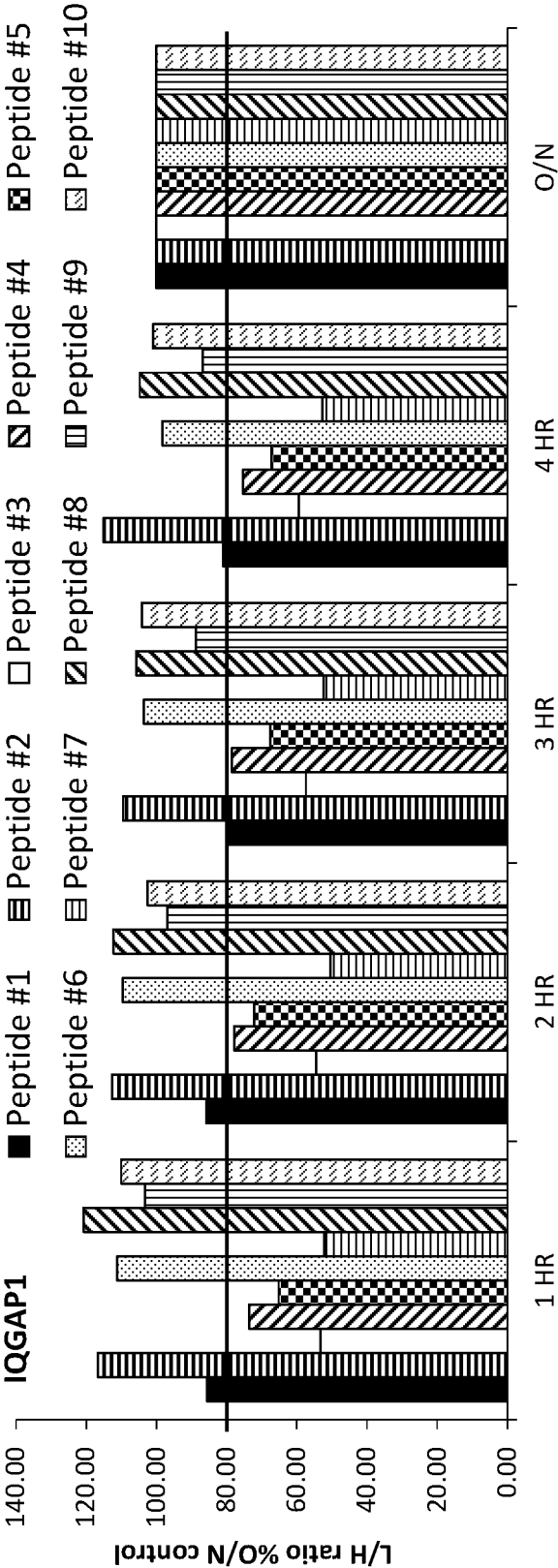


Figure 12F

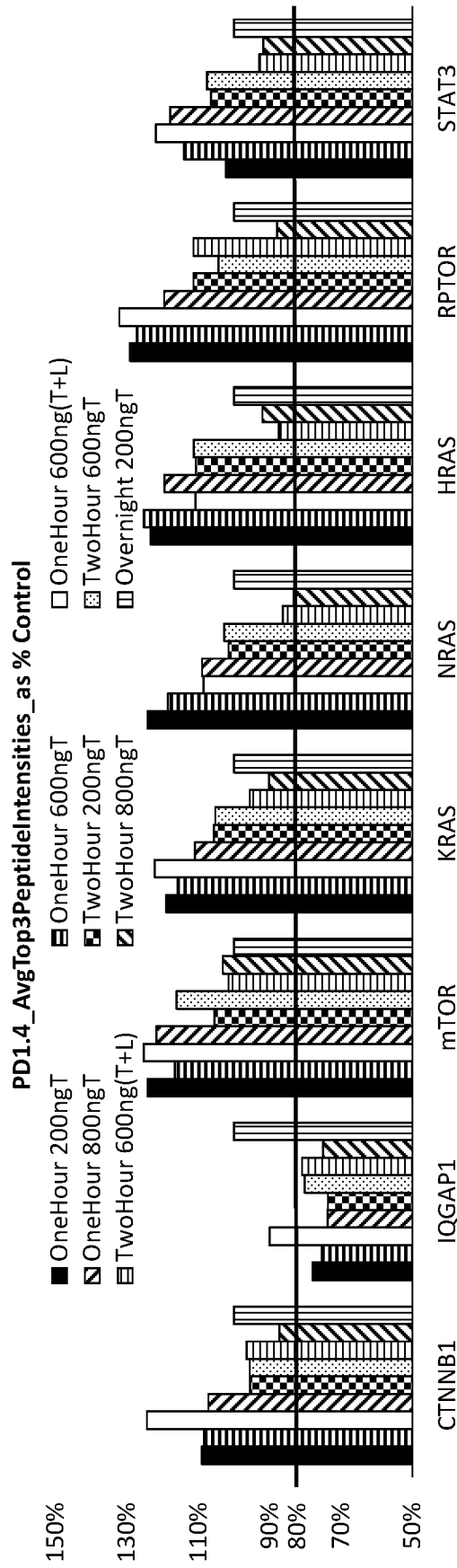


Figure 13A

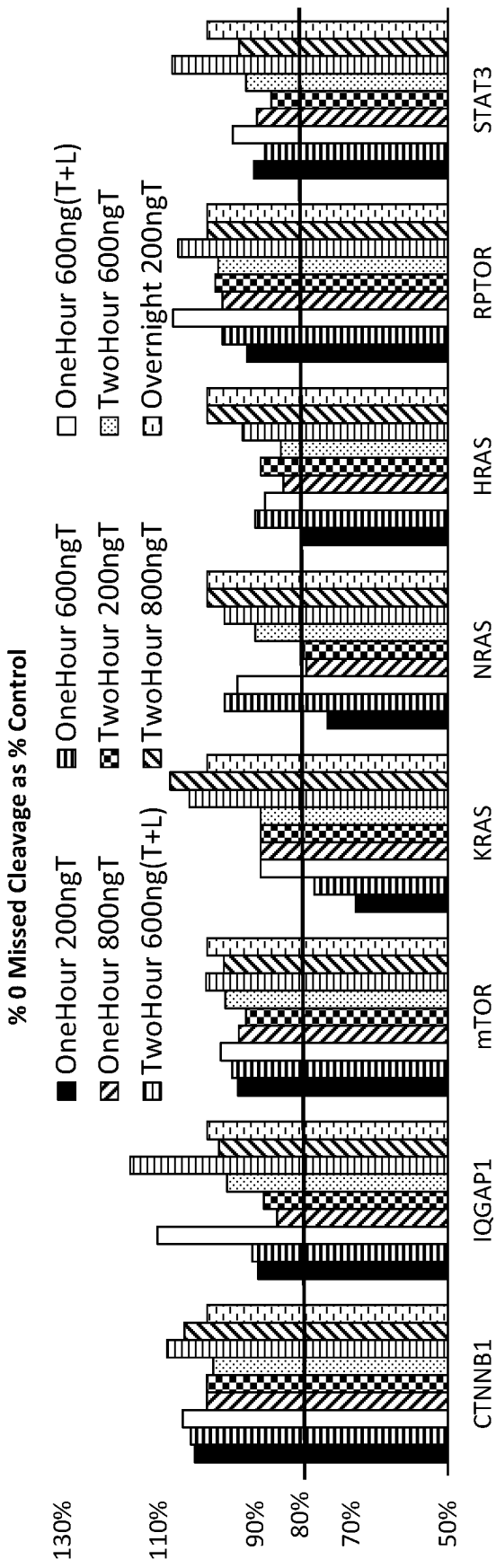


Figure 13B

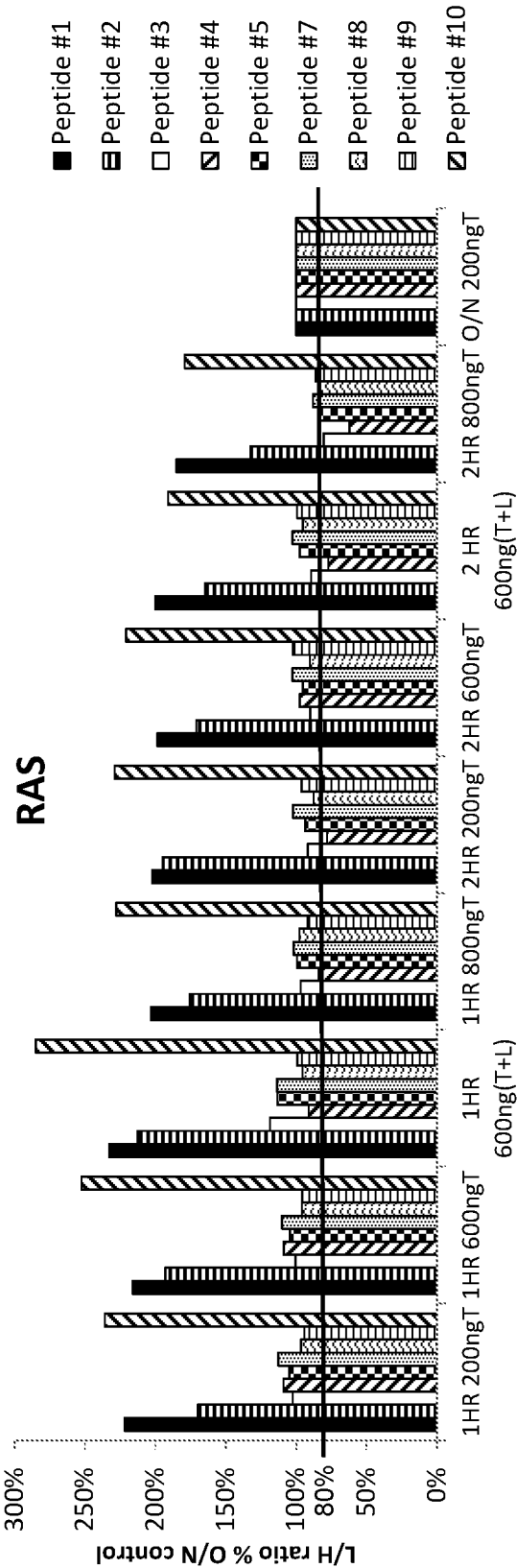


Figure 14A

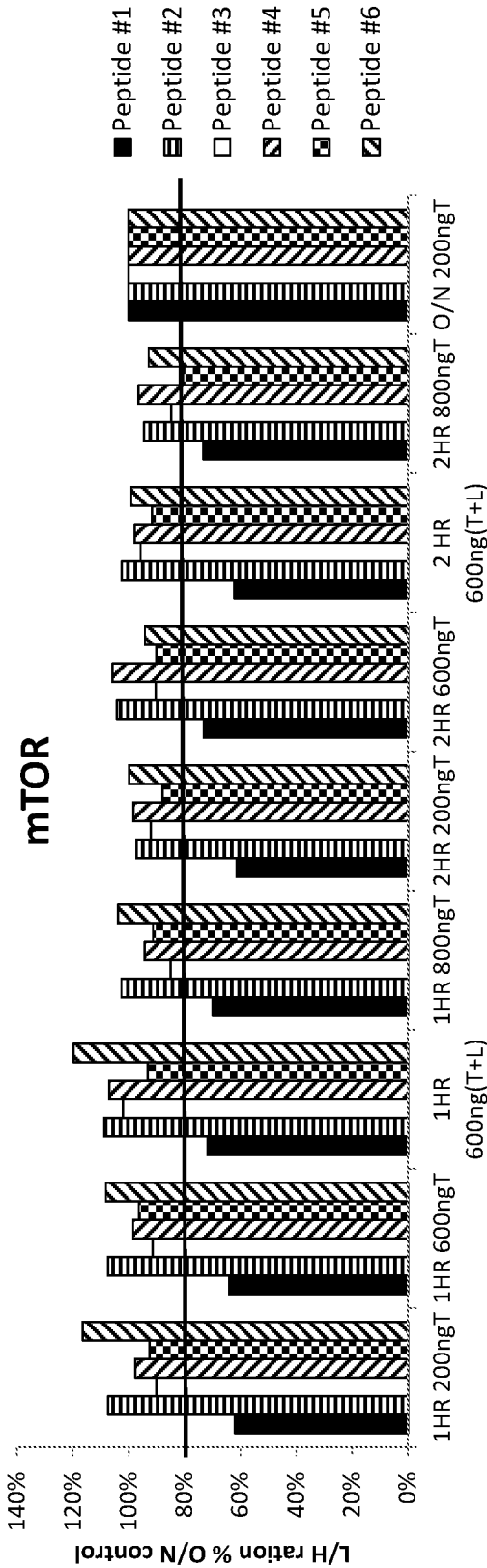


Figure 14B

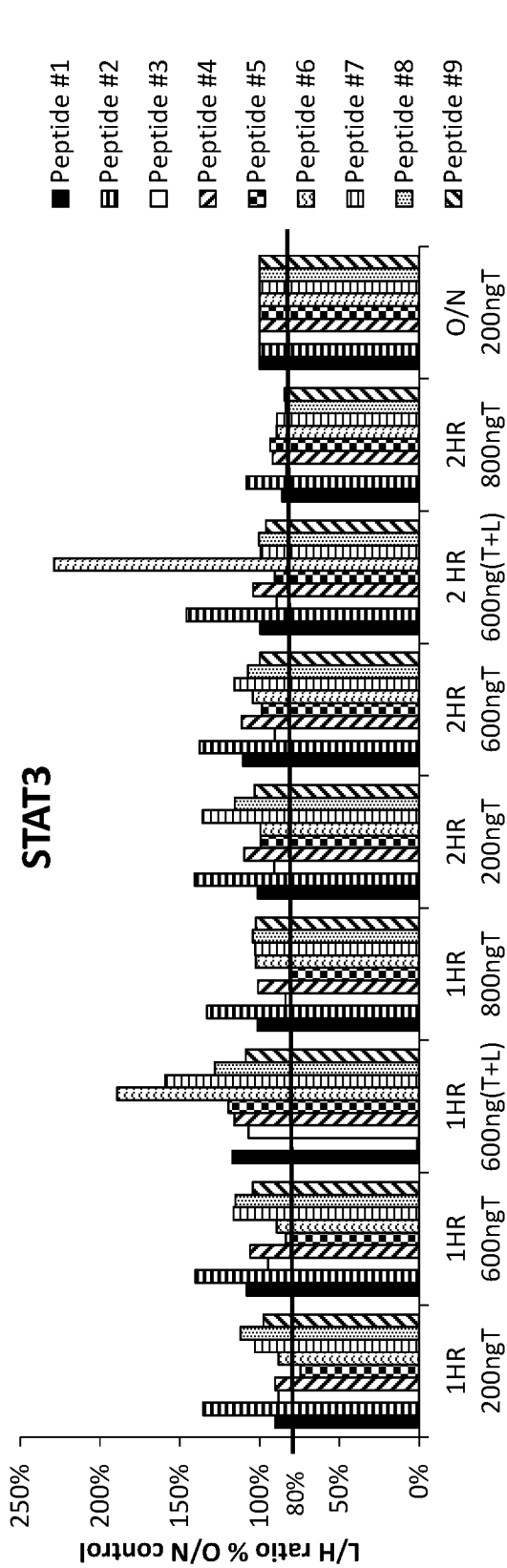


Figure 14C

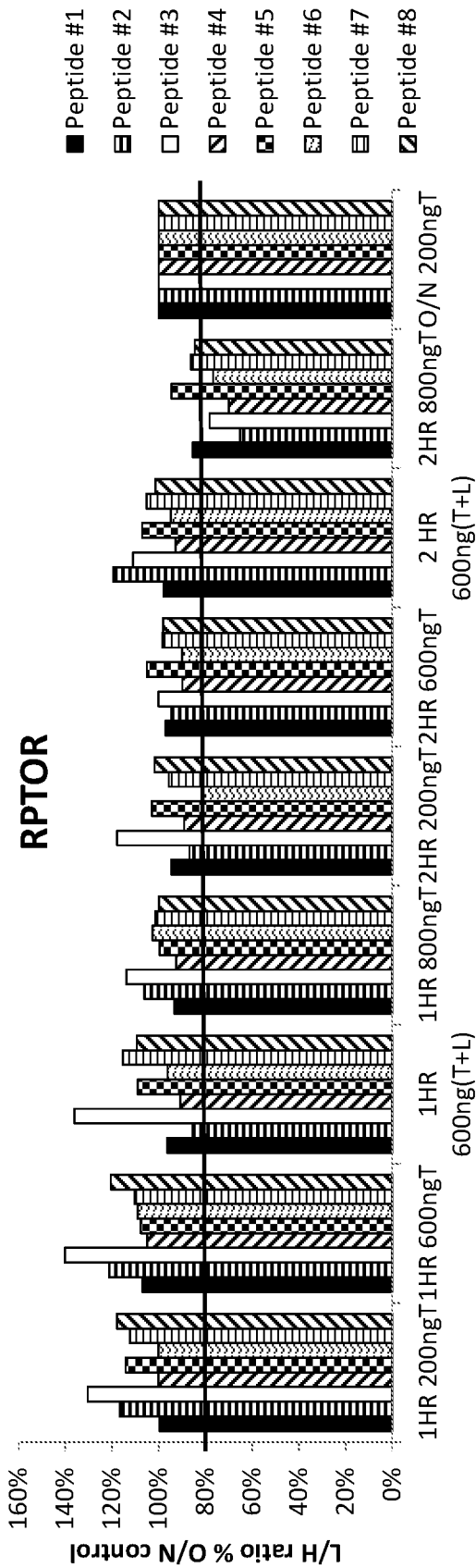


Figure 14D

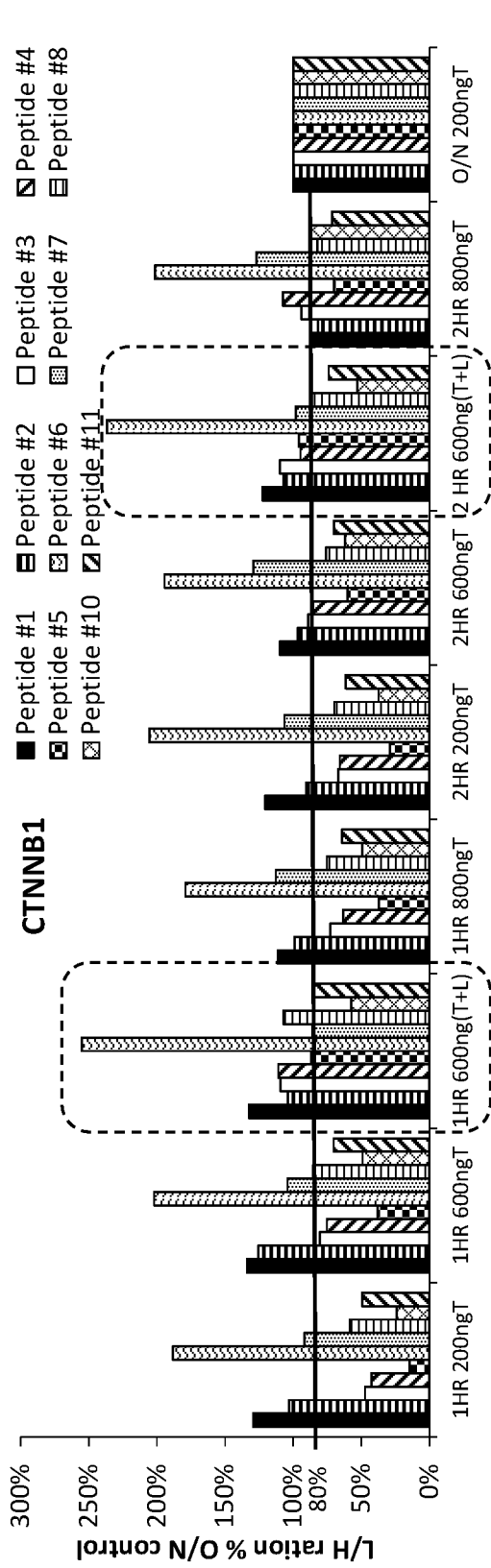


Figure 14E

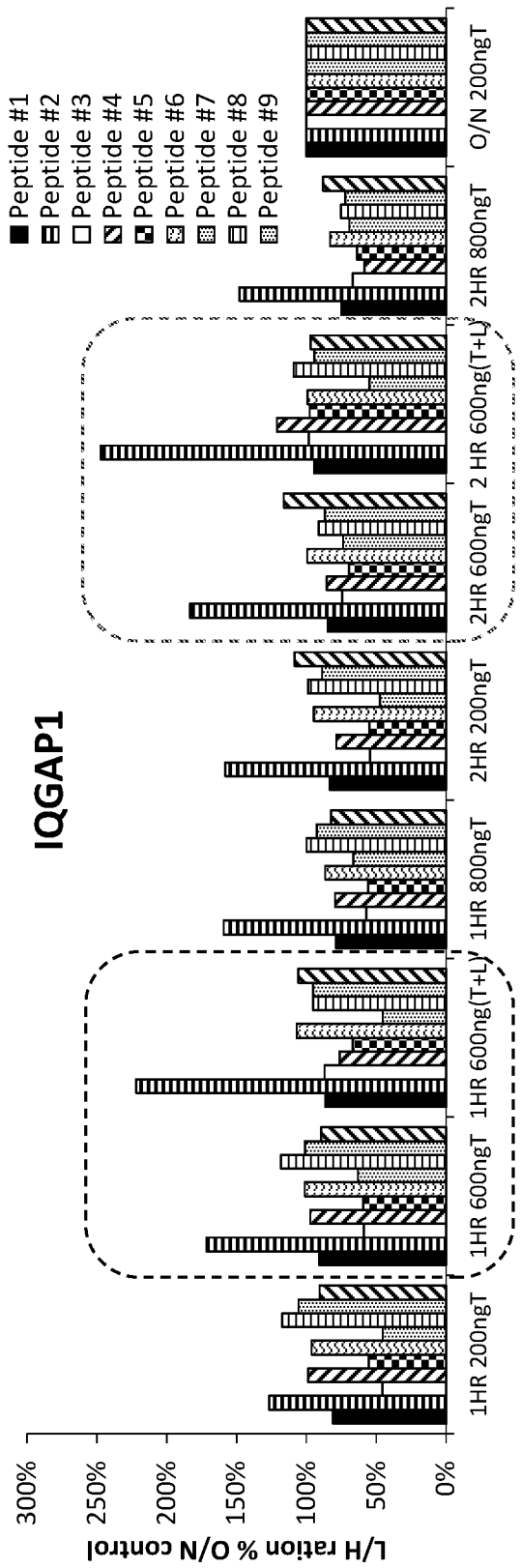


Figure 14F

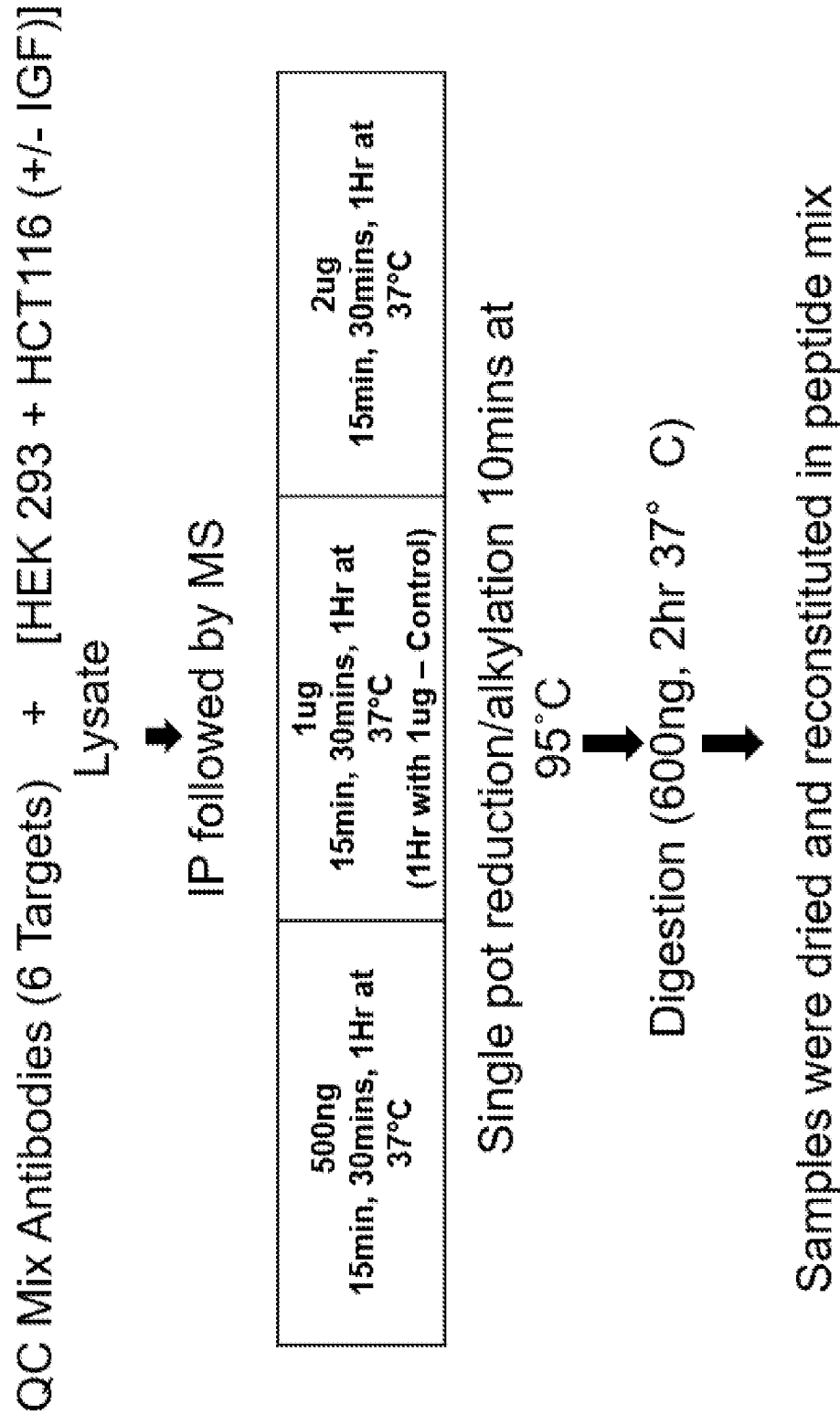
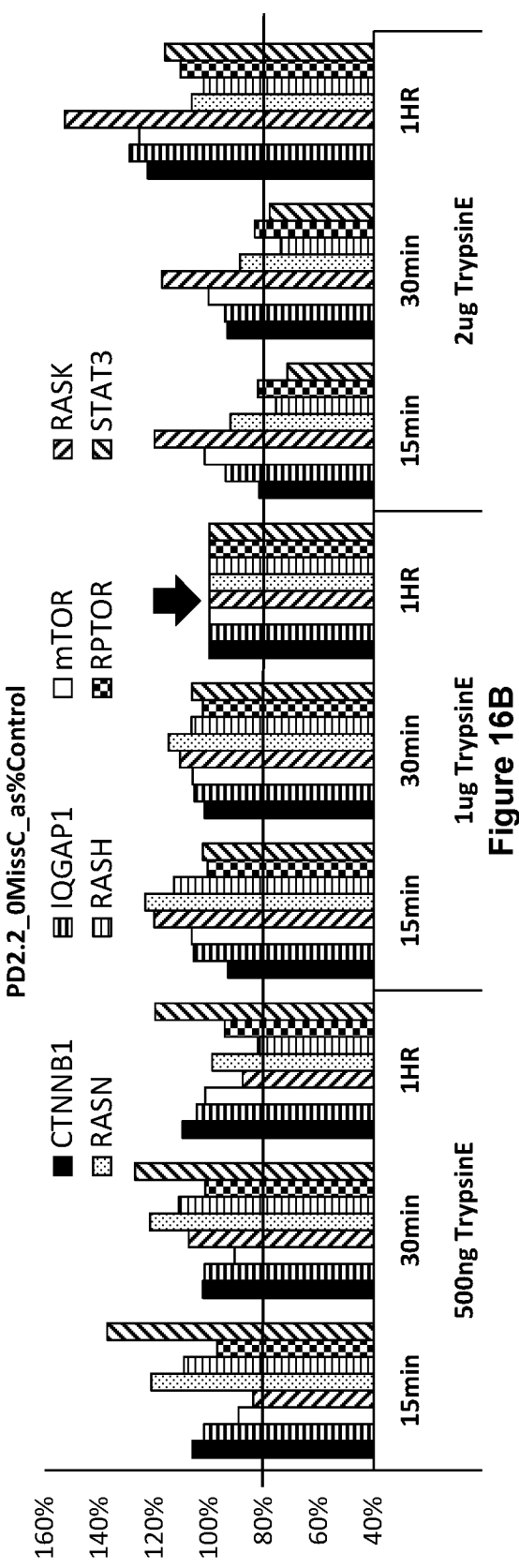
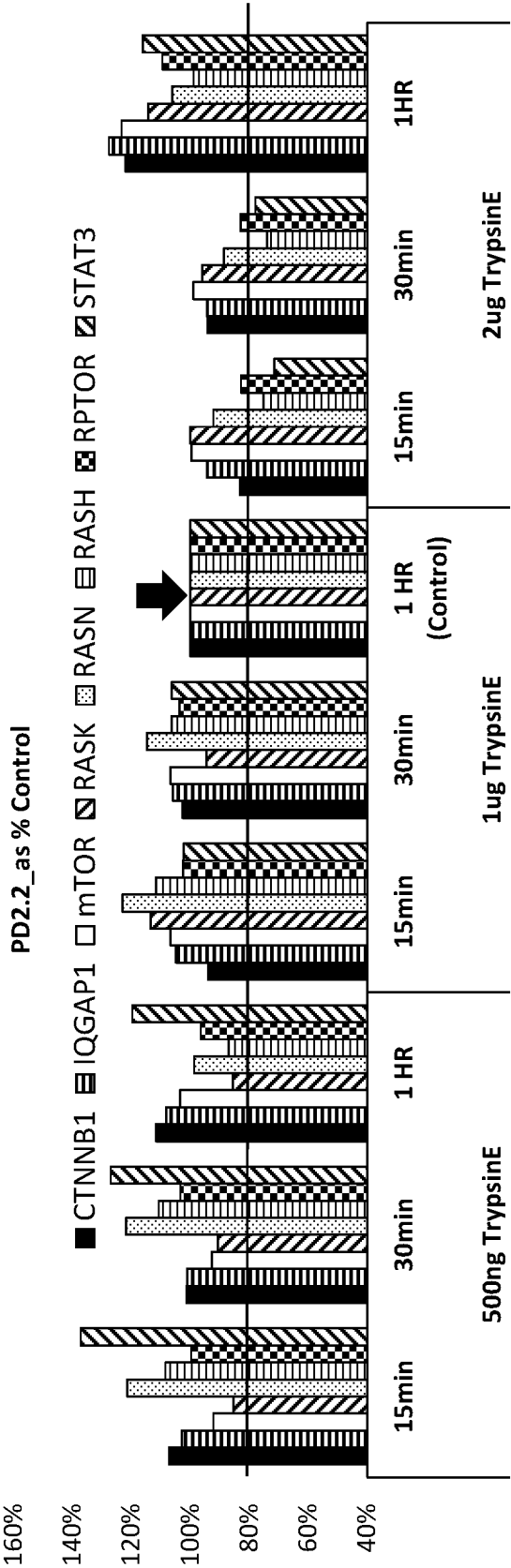


Figure 15



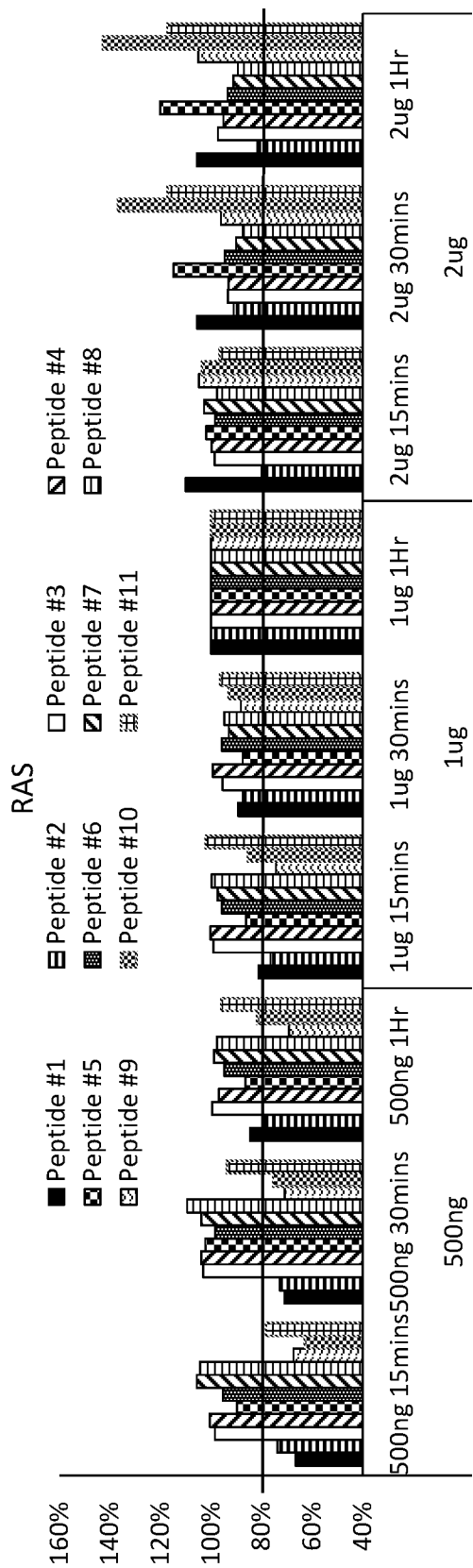


Figure 17A

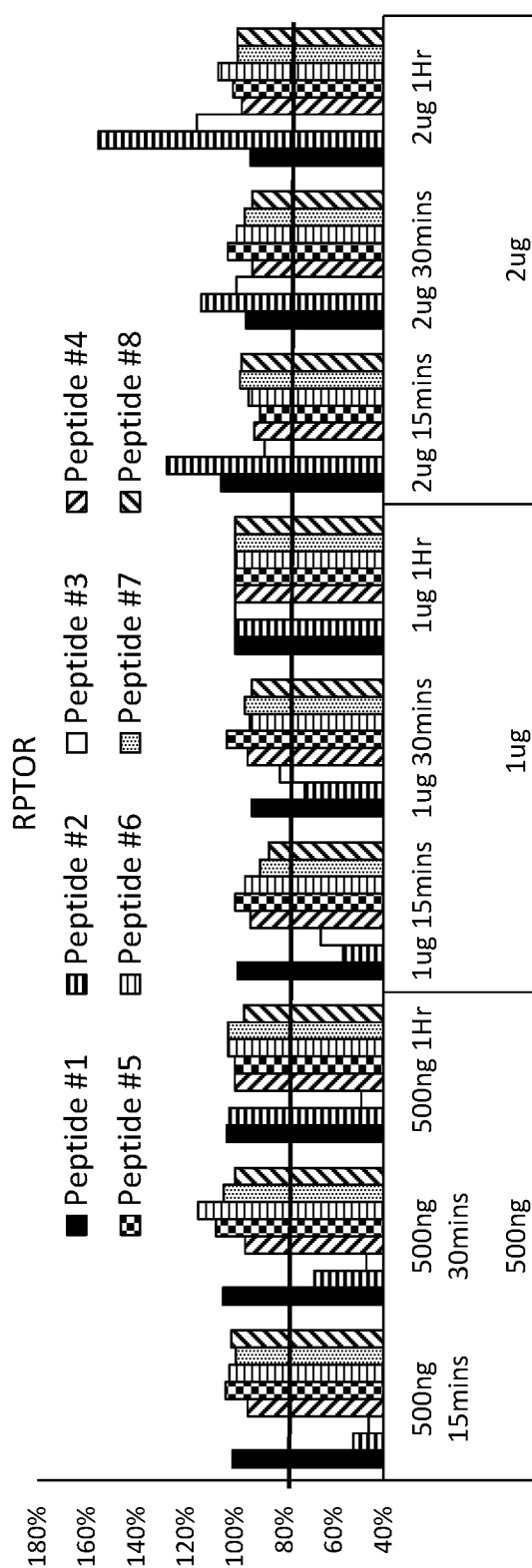
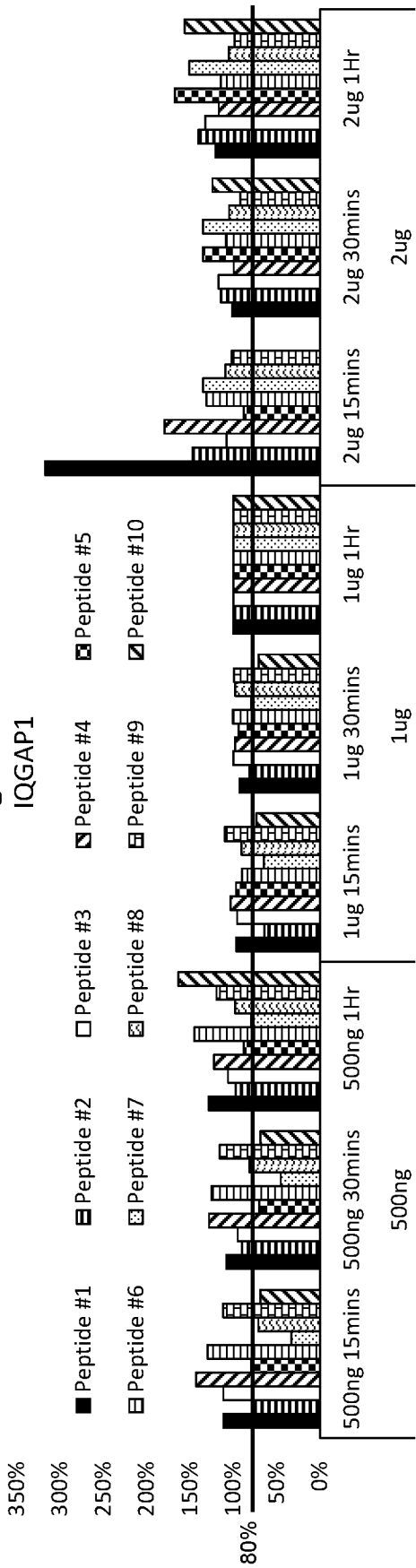
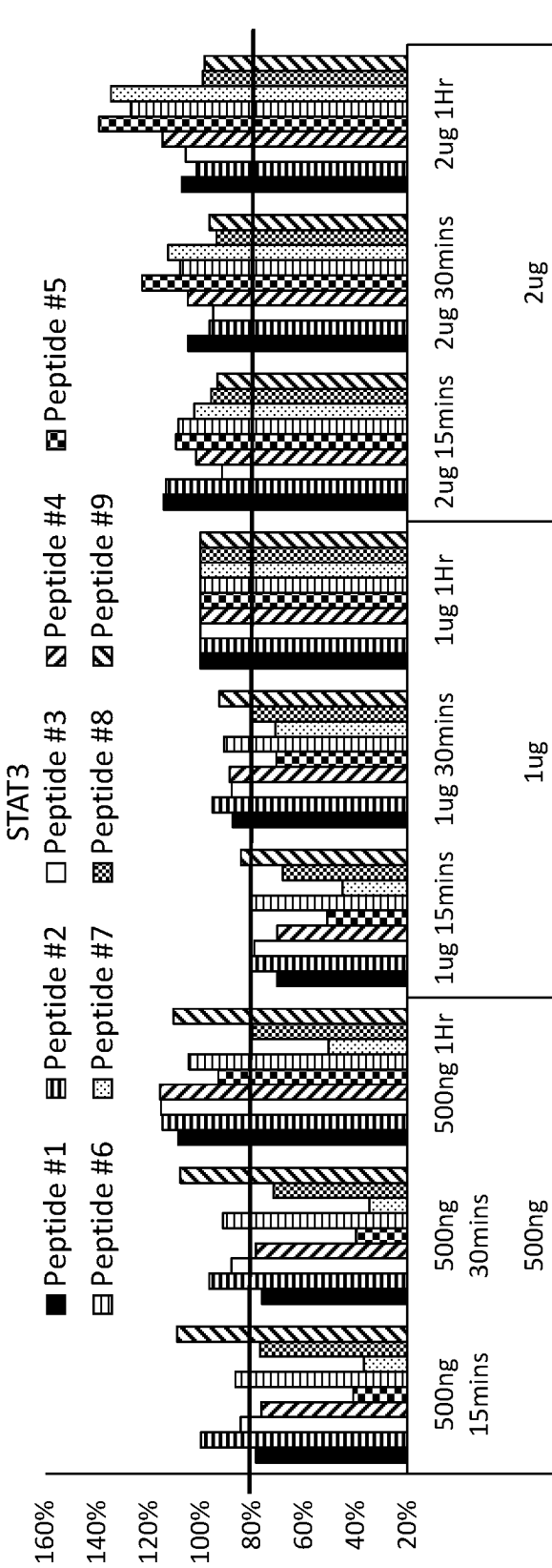


Figure 17B



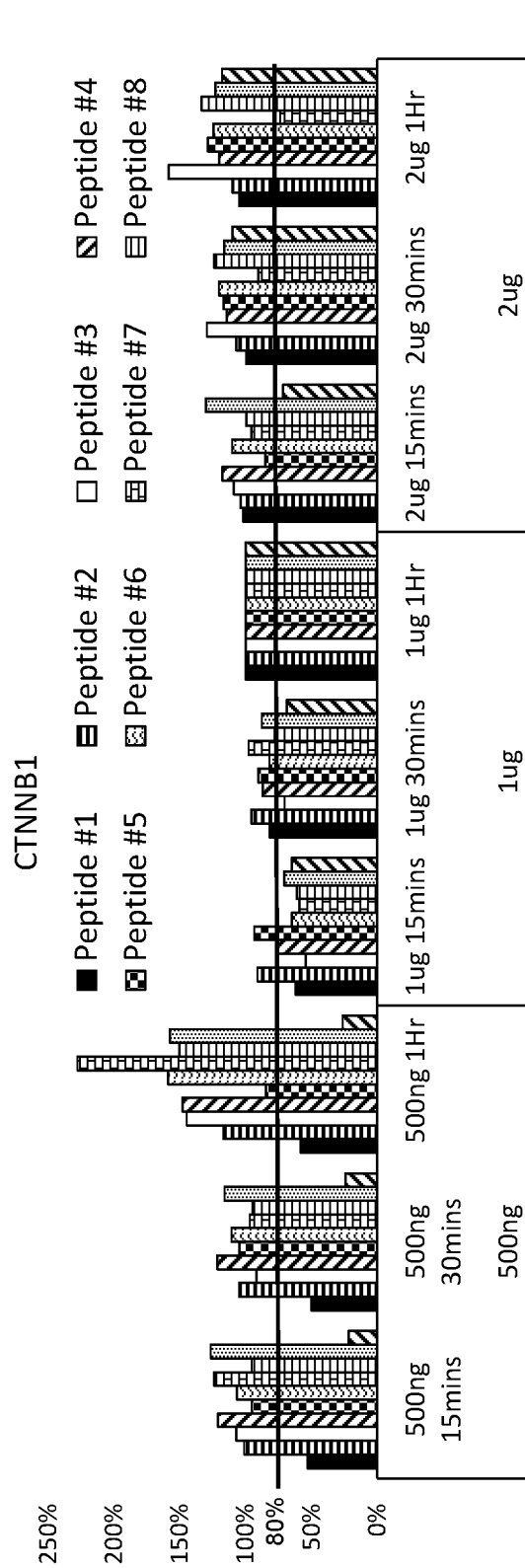


Figure 17E

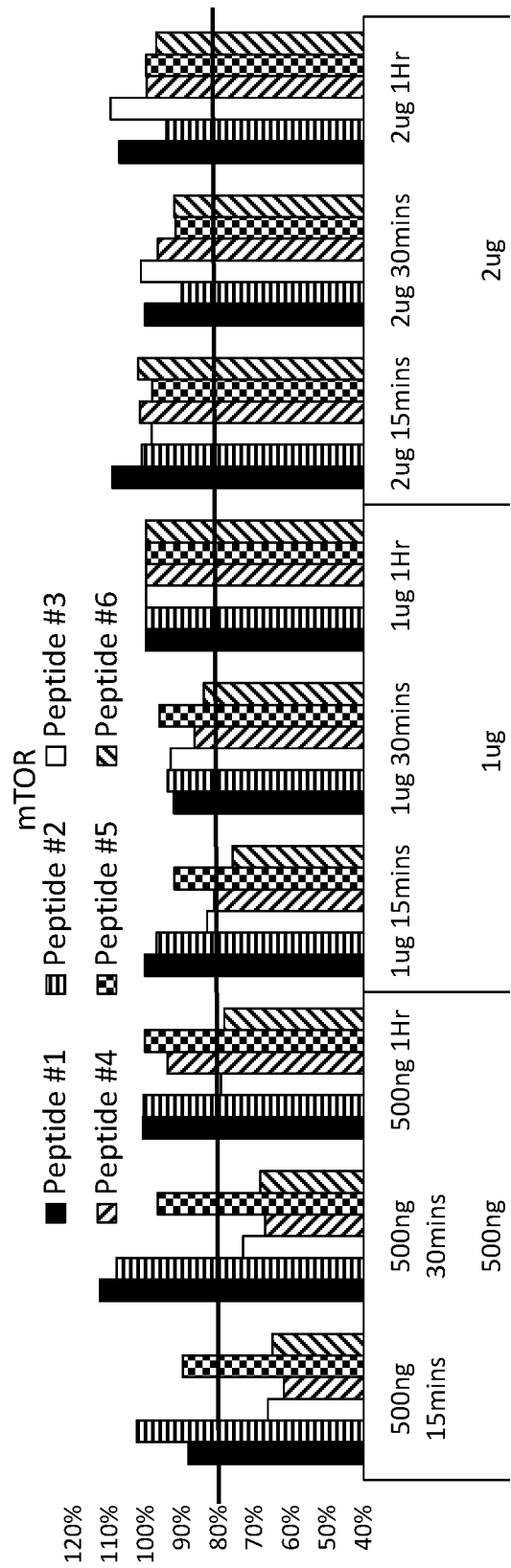


Figure 17F

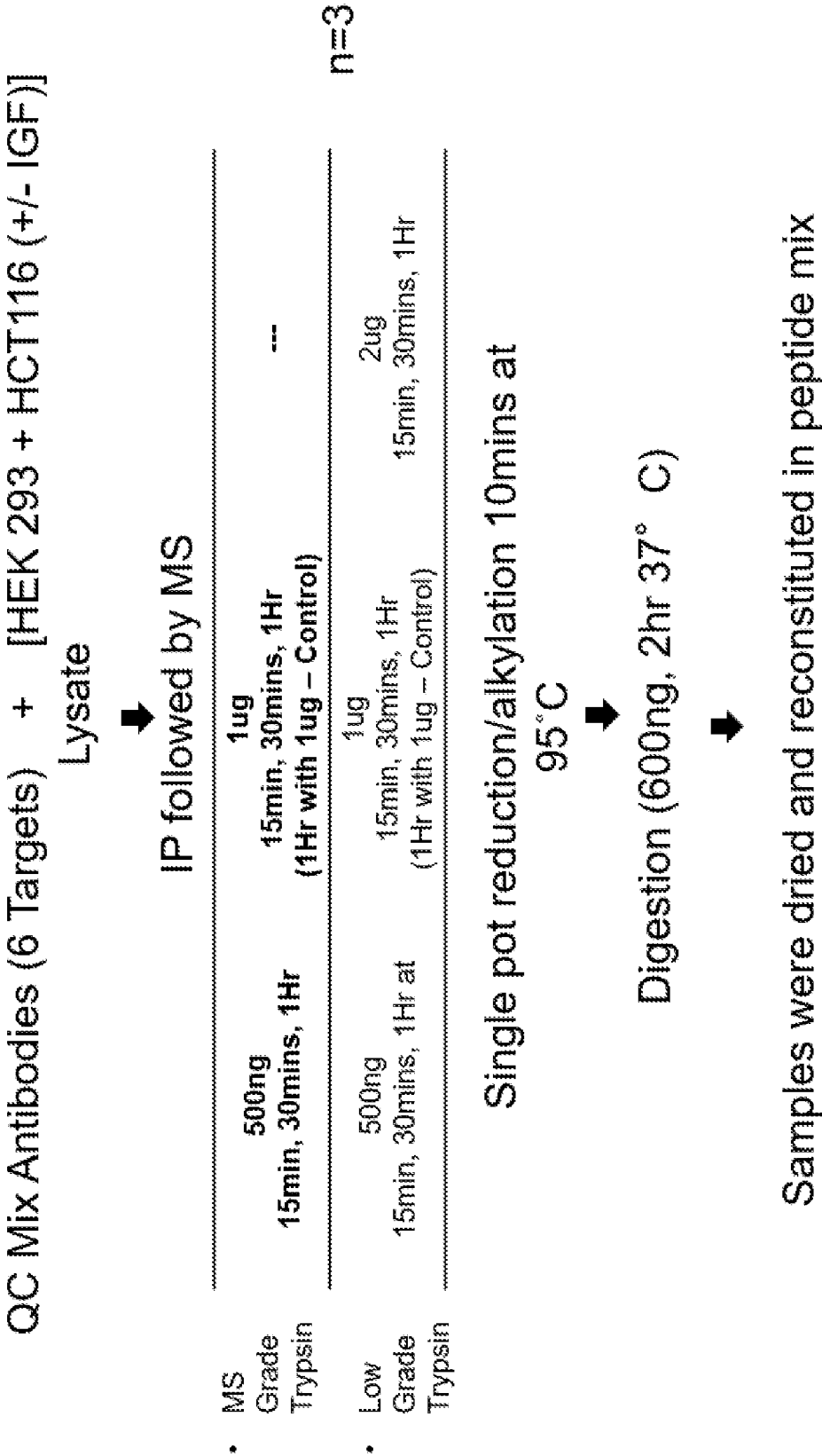


Figure 18

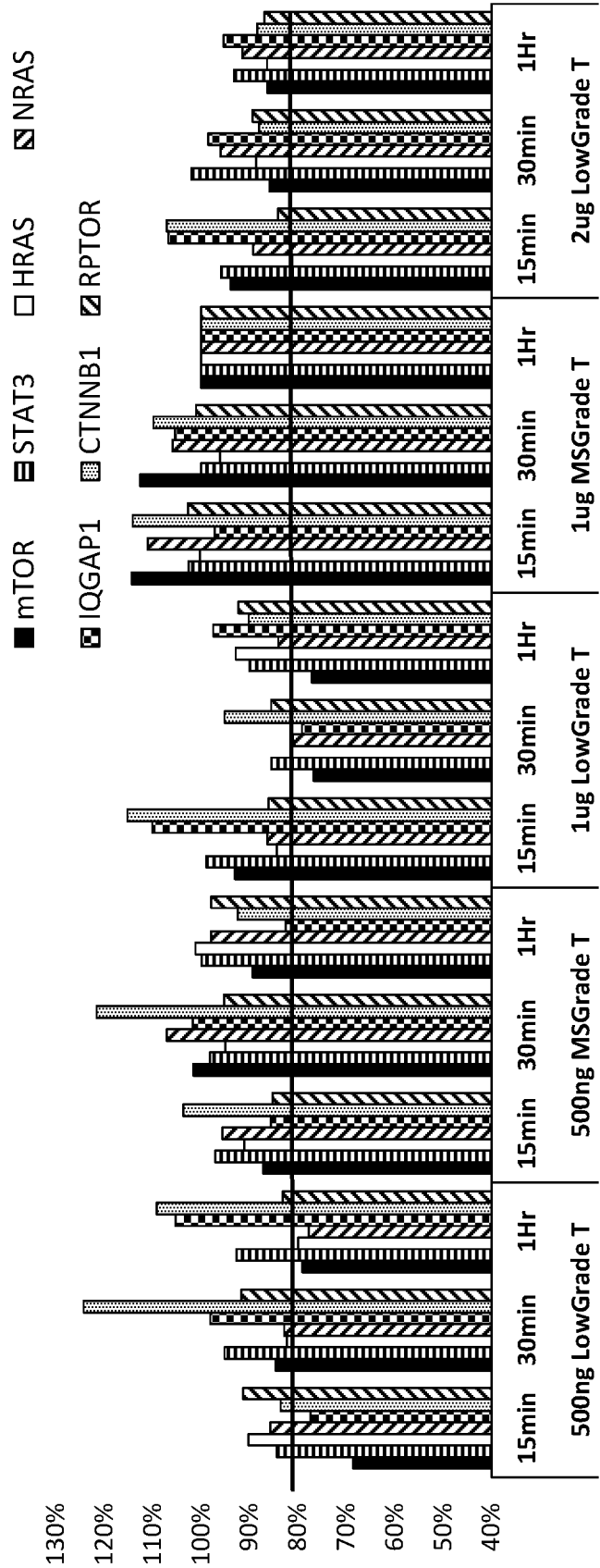


Figure 19A

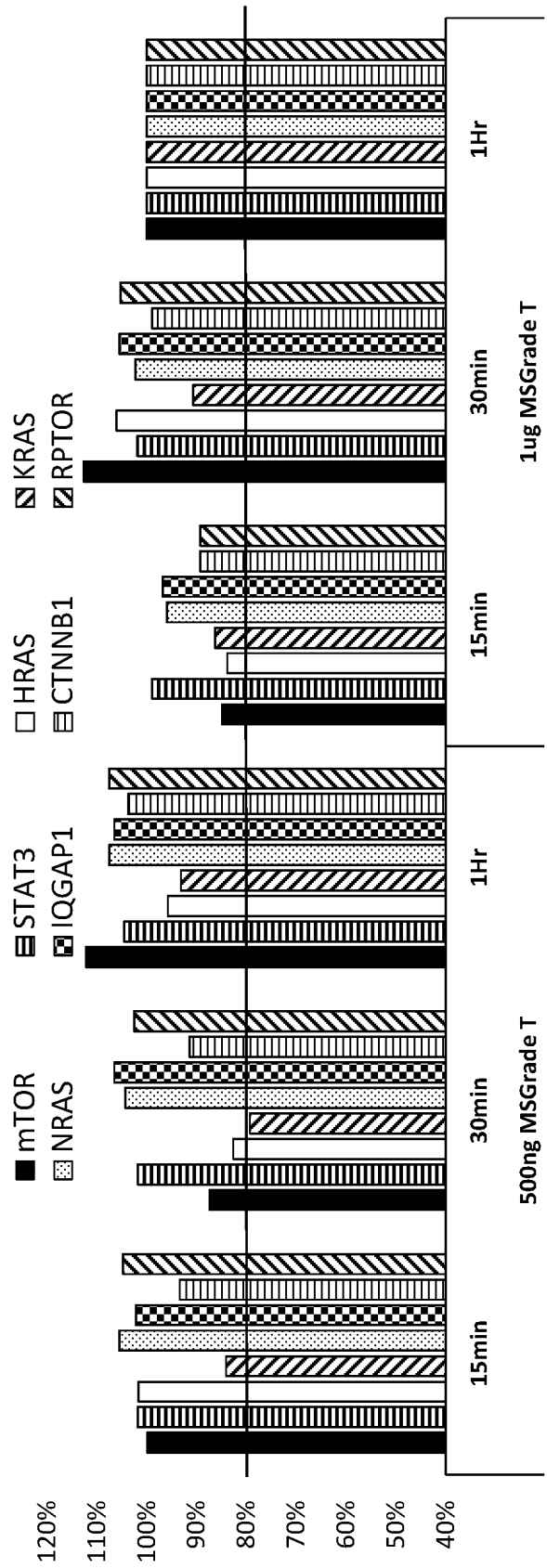


Figure 19B

%CVs	0.5ug 15min	0.5ug 30min	0.5ug 1Hr	1ug 15min	1ug 30min	1ug 1Hr	2ug 15min	2ug 30min	2ug 1Hr
CTNNB1	13%	21%	26%	2%	25%	24%	9%	33%	22%
IQGAP1	15%	20%	22%	11%	21%	12%	9%	26%	8%
mTOR	15%	20%	22%	6%	17%	11%	4%	22%	8%
RASK	19%	29%	26%	14%	24%	2%	7%	24%	10%
RASN	8%	17%	11%	11%	11%	4%	7%	18%	3%
RASH	6%	12%	6%	8%	12%	2%	15%	25%	4%
RPTOR	7%	14%	13%	9%	16%	7%	10%	24%	3%
STAT3	5%	8%	18%	9%	14%	8%	16%	31%	12%

Figure 20A

%CVs	0.5ug 15min	0.5ug 30min	0.5ug 1Hr	1ug 15min	1ug 30min	1ug 1Hr	2ug 15min	2ug 30min	2ug 1Hr
CTNNB1	13%	21%	26%	2%	25%	24%	9%	33%	22%
IQGAP1	15%	20%	22%	11%	21%	12%	9%	26%	8%
mTOR	15%	20%	22%	6%	17%	11%	4%	22%	8%
RASK	19%	29%	26%	14%	24%	2%	7%	24%	10%
RASN	8%	17%	11%	11%	11%	4%	7%	18%	3%
RASH	6%	12%	6%	8%	12%	2%	15%	25%	4%
RPTOR	7%	14%	13%	9%	16%	7%	10%	24%	3%
STAT3	5%	8%	18%	9%	14%	8%	16%	31%	12%

Figure 20B

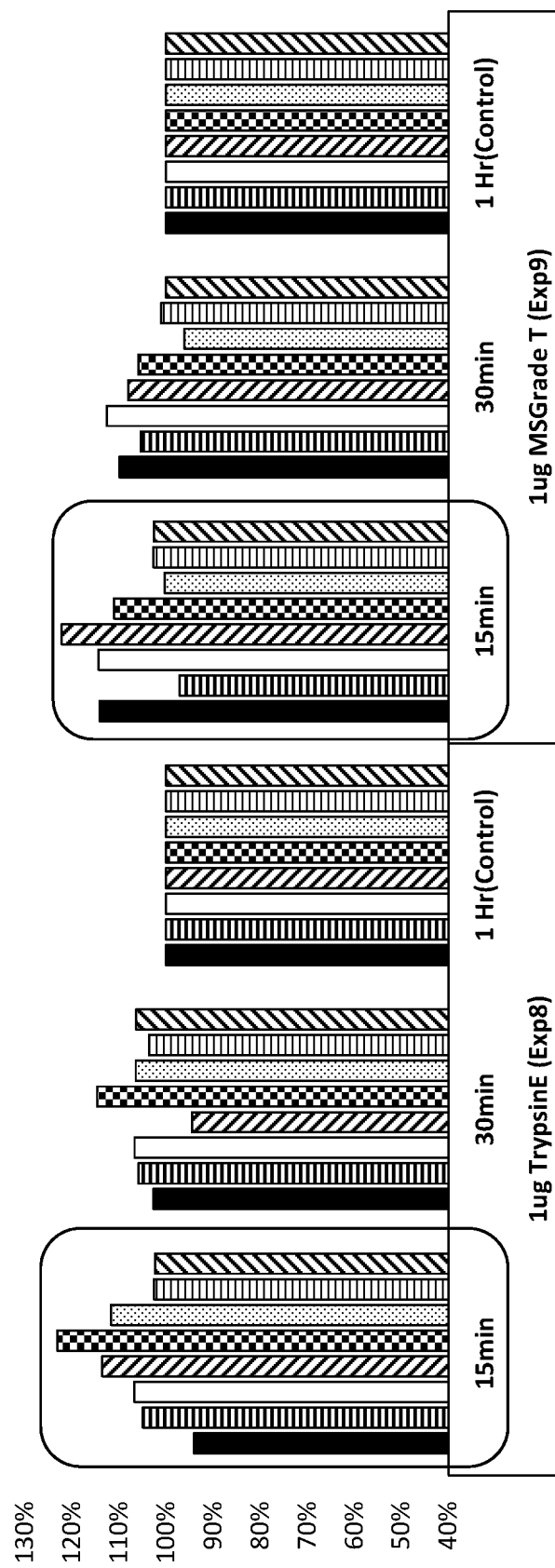


Figure 21

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2019/019577A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/68
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, WPI Data, EMBASE, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2017/160698 A1 (PIERCE BIOTECHNOLOGY INC [US]) 21 September 2017 (2017-09-21) Whole document, in particular claims and figure 2 ----- -/--	4,8, 13-17



Further documents are listed in the continuation of Box C.



See patent family annex.

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"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

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Date of the actual completion of the international search

3 June 2019

Date of mailing of the international search report

18/06/2019

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
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Fax: (+31-70) 340-3016

Authorized officer

Vanmontfort, D

INTERNATIONAL SEARCH REPORT

International application No

PCT/US2019/019577

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	AGNES RAFALKO ET AL: "Immunoaffinity Enrichment and Liquid Chromatography-Selected Reaction Monitoring Mass Spectrometry for Quantitation of Carbonic Anhydrase 12 in Cultured Renal Carcinoma Cells", ANALYTICAL CHEMISTRY, vol. 82, no. 21, 1 November 2010 (2010-11-01), pages 8998-9005, XP055593178, US ISSN: 0003-2700, DOI: 10.1021/ac101981t cited in the application whole document, in particular figure 1; materials and methods -----	9,10
X	BENEDETTA TURRIZIANI ET AL: "On-Beads Digestion in Conjunction with Data-Dependent Mass Spectrometry: A Shortcut to Quantitative and Dynamic Interaction Proteomics", BIOLOGY, vol. 3, no. 2, 16 April 2014 (2014-04-16), pages 320-332, XP055593233, DOI: 10.3390/biology3020320	1-3,5-7, 11,12, 18,19
Y	whole document, in particular abstract; experimental section; paragraphs 3.1-3.3; Figures 1 and 2 -----	4,8-10, 13-17

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/US2019/019577

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2017160698 A1	21-09-2017	CN 108885215 A	23-11-2018
		EP 3430405 A1	23-01-2019
		US 2019086420 A1	21-03-2019
		WO 2017160698 A1	21-09-2017
