

# Detection of Cellular Response to an in vitro Challenge with Bacterial Gram-Negative Lipopolysaccharides (LPS) in Peripheral Blood Mononuclear Cells (PBMCs) for Biomarker Research

David Sarracino,<sup>1</sup> Jennifer N Sutton,<sup>1</sup> Maryann S Vogelsang,<sup>1</sup> Bryan Krastins,<sup>1</sup> Gregory Byram,<sup>1</sup> Amol Prakash,<sup>1</sup> Vineet Gupta,<sup>2</sup> Mary F Lopez<sup>1</sup>

<sup>1</sup>Thermo Fisher Scientific, BRIMS (Biomarker Research Initiatives in Mass Spectrometry), Cambridge, MA;

<sup>2</sup>Rush University Medical Center, Chicago, IL



After incubation at 37 °C for 30 min the cells were isolated according to the manufacturer's instructions for a total exposure time of 60 min. Rinsed cell pellets (~2 mg) were denatured in 350  $\mu$ L of 8M Urea 300 mM Tris-HCl 2.5% n-propanol 10 mM Dithiothreitol, reduced/alkylated, diluted to 2 mL with 50 mM Tris, 5 mM CaCl<sub>2</sub> and digested overnight with 20  $\mu$ g of Pierce Trypsin Protease, MS Grade.

### Liquid Chromatography

Peptide retention time standards were added and the samples were loaded into 96-well plates onto a Thermo Scientific™ EASY-nLC™ 1000. Separations were done on a Thermo Scientific™ Dionex™ PS-DVB trap column, (5  $\mu$ m particle, 300Å pore, 150  $\mu$ m x 12 cm) connected in a "vented t" configuration to a 5  $\mu$ m particle, 200Å pore C18AQ 100  $\mu$ m x 50 cm packed tip resolving column in a Thermo Scientific™ Nanospray Flex™ Ion Source on a hybrid Thermo Scientific™ Orbitrap Velos Pro™ MS. Stepped Flow and gradient from 4-50%B at 650 nL/min over 205 min. Buffer A is 2% Methanol 0.2% formic acid, water(v/v). Buffer B is 10% water, 10% isopropanol, 80% acetonitrile 0.2% formic acid (v/v), all solvents are Thermo Scientific™ Optima™ LC-MS grade.

Portions of each of the TLR4 digests (Low and High stim) pooled for library creation were fractionated into 12 fractions of 1.8 ml, on a 4.6 mm x 25 cm PS-DVB column 8  $\mu$ m particle 300Å pore, buffer A: 100 mM ammonium formate, 58 mM ammonium hydroxide, Buffer B: 29 mM ammonium hydroxide in 91% acetonitrile 9% water (v/v), using a flow rate of 1 mL/min in a gradient to 45% B (Figure 2).

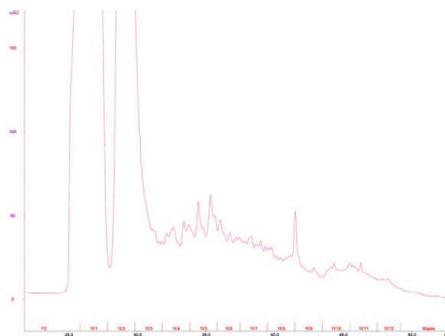
### Mass Spectrometry

For mass spectrometric analysis, a data-dependent top 25 method has been used. Full MS scans acquired at a resolution of 100,000 using a 1e6 target value, with dependent scans analyzed in the linear ion trap with normal scan resolution. Uncharacterized charge states and + 1 charge states are rejected. Chromatography phase triggering with monoisotopic fitting was used with a peak width of 40 s and a minimum peak threshold of 3.5e4. The maximum inject time allowed for MS/MS scans was set to 100 ms. Dynamic exclusion is turned on using a peak width of 60 s.

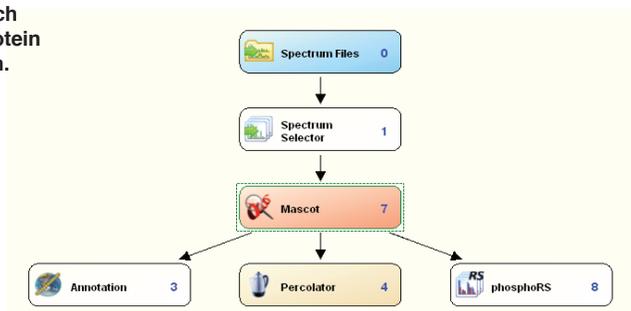
### Data Analysis

Full-scan comparisons were made using Pinpoint software, and MS/MS spectra were processed by Proteome Discoverer software using The Mascot® search engine. Two different peptide identification strategies were used. The simple search method (Figure 3) only searches for high-confidence, tryptic peptides and phosphopeptides. The more complex search strategy (Figure 4), breaks the PTM search strategy into multiple nodes, where small groups of PTMs, likely to occur on the same peptide, are searched in each node. This allows for higher-confidence assignments due to the reduced size of each database, albeit at an increased search computational time. Pathway information was processed using Thermo Scientific™ ProteinCenter™ software (not shown). Pinpoint software allows for the import of spectral libraries which can be obtained from data from both unfractionated and fractionated samples provided the chromatography in all samples is reproducible and retention times are consistent.

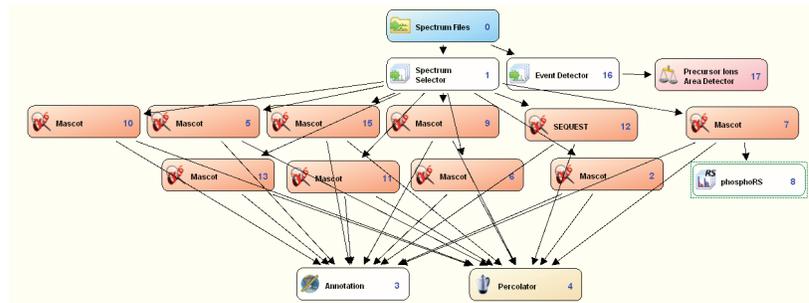
**FIGURE 2. High pH reverse phase fractionation for library peptide fractionation**



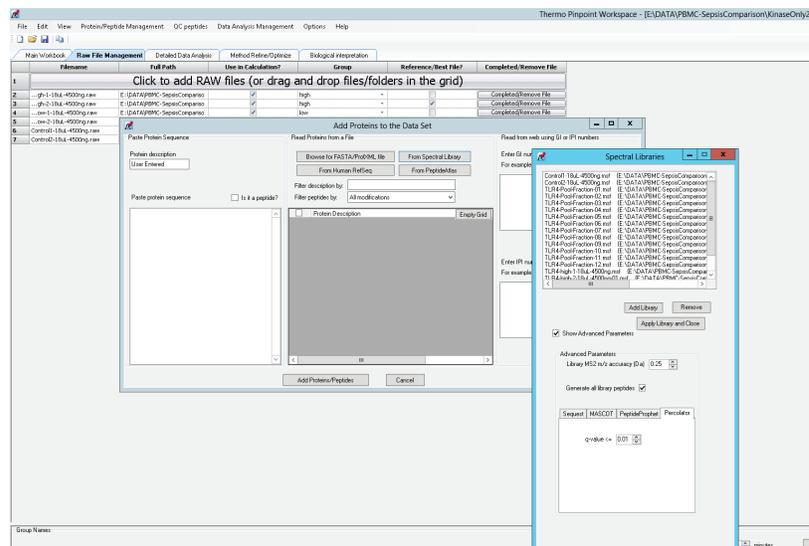
**FIGURE 3. Search workflow for protein phosphorylation.**



**FIGURE 4. Search workflow for multiple modifications. Searches are broken up into groups of most likely to occur modifications. This search strategy is computationally intensive and works best with the high-resolution Orbitrap-analyzed MS2. Many modifications such as ubiquitination, oxidations and deamidations, semi-tryptic, and different databases can be searched even by other search engines without compromising the integrity of the results.**



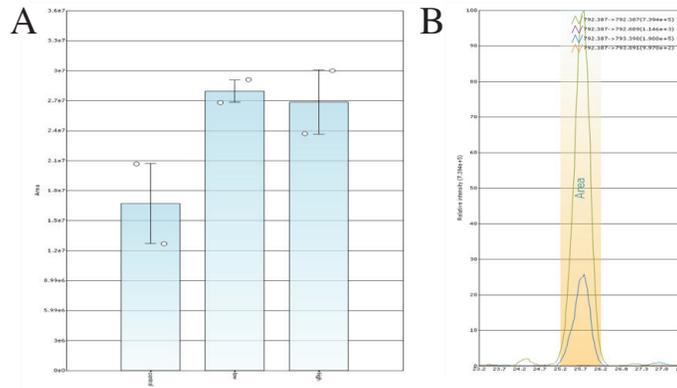
**FIGURE 5. Results from different search strategies and any fractionation can then be brought into Pinpoint software through the spectral library function.**



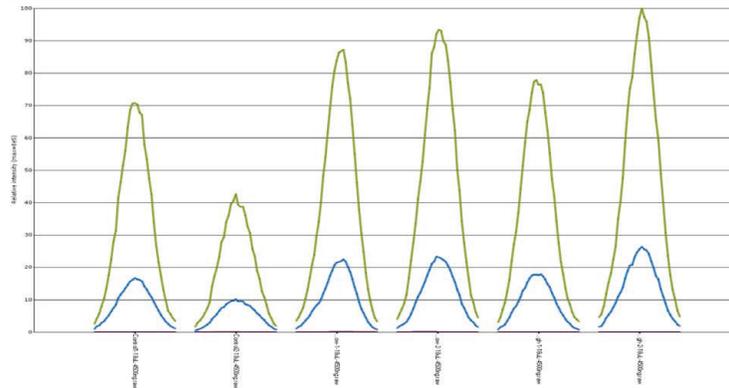
## Results

In order to allow the detection of differentially expressed proteins and peptides, instrumentation should provide enough quantitative full-scan measurements while simultaneously providing MS/MS fragmentation data to allow sequencing of as many peptides as possible. In this experiment, ca 4000 proteins were identified over 95% confidence containing >250 phosphorylations, >150 ubiquitylations, peptide oxidations (other than methionine) that were inserted into the Pinpoint spectral library. Pinpoint software has the advantage in that it allows for a selected protein/peptide library import. In this example, all kinases identified in the library can be selected for analysis. Once selected, proteins that are up or down regulated can be highlighted and verified using Pinpoint software (Figures 6, 7). In addition to protein class selection, specific proteins of interest (Figure 8) TGF beta and pathways specific components, (Figure9) MAPKKK 15, can be selected and analyzed for quantification.

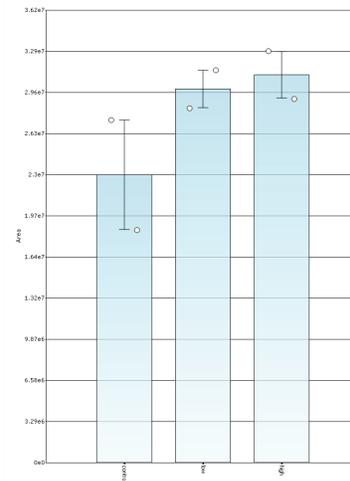
**FIGURE 6. Quantification of the +2 charge state of peptide GDDTPLHLAASHGHR from integrin-linked protein kinase gil4758606 [Homo sapiens] A) Comparison of Control, Low and High Stimulation B) Alignment of all isotopes.**



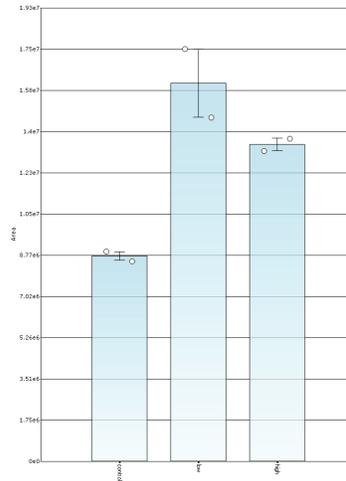
**FIGURE 7. View of the replicates of sample quantifications from the first two isotopes (792.387, green: 792.889, blue) of the peptide GDDTPLHLAASHGHR in Control, Low and High stimulation experiments (pairs, left to right).**



**FIGURE 8. Signal Pathway proteins up-regulated from LPS stimulation. Peptide GGEIEGFR from transforming growth factor beta-1 precursor gil63025222 [Homo sapiens].**



**FIGURE 9. MyD88 signal pathway proteins up-regulated from LPS stimulation. Peptide QILEGLK from mitogen-activated protein kinase kinase kinase 15 gii282847398 [Homo sapiens].**



## Conclusion

- The *in vitro* whole blood PBMC stimulation model combined with a simple sample preparation workflow strategy allows for a facile and reproducible method of testing cell signaling in the immune response from human research samples.
- Spectral Library creation from simple fractionation techniques allows for a large number of different PTMs to be identified without using any specific PTM enrichment strategy.
- Pinpoint software allows for targeted quantification in even full scan discovery modes in complex sample mixtures.
- Future work will be to utilize the library and initial data to generate a targeted list of peptides for selected reaction monitoring (SRM) workflows on both Thermo Scientific™ TSQ Vantage™ and Thermo Scientific™ Q Exactive™ instruments. These will be to address the low level library peptides that were difficult to quantify in this workflow, but are of interest in the biological pathways that were shown to be up- and down-regulated in these samples and are also correlated with sepsis.

## References

1. Angus DC, Linde-Zwirble WT, Lidicker J, Clermont G, Critical Care Med 2001, (29), 1303-1310
2. Robbins Basic Pathology. 8th edition
3. Mendes ME, Baggio-Zappia GL, Brunialti MK, Fernandes Mda L, Rapozo MM, Salomao R. Immunobiology, 2011 Mar;216(3):285-95.

[www.thermofisher.com](http://www.thermofisher.com)

©2016 Thermo Fisher Scientific Inc. All rights reserved. Mascot is a registered trademark of Matrix Science. Vacutainer is a trademark of Becton Dickinson. All other trademarks are the property of Thermo Fisher Scientific, Inc. and its subsidiaries. Specifications, terms and pricing are subject to change. Not all products are available in all countries. Please consult your local sales representative for details.

**Africa-Other** +27 11 570 1840  
**Australia** +61 3 9757 4300  
**Austria** +43 1 333 50 34 0  
**Belgium** +32 53 73 42 41  
**Canada** +1 800 530 8447  
**China** +86 10 8419 3588  
**Denmark** +45 70 23 62 60

**Europe-Other** +43 1 333 50 34 0  
**Finland/Norway/Sweden**  
+46 8 556 468 00  
**France** +33 1 60 92 48 00  
**Germany** +49 6103 408 1014  
**India** +91 22 6742 9434  
**Italy** +39 02 950 591

**Japan** +81 45 453 9100  
**Latin America** +1 561 688 8700  
**Middle East** +43 1 333 50 34 0  
**Netherlands** +31 76 579 55 55  
**New Zealand** +64 9 980 6700  
**Russia/CIS** +43 1 333 50 34 0  
**South Africa** +27 11 570 1840

**Spain** +34 914 845 965  
**Switzerland** +41 61 716 77 00  
**UK** +44 1442 233555  
**USA** +1 800 532 4752

**Thermo**  
SCIENTIFIC

Part of Thermo Fisher Scientific