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This is the author's accepted version of the contribution published as:

*Original*

Targeted quantitation of HMGB1 protein by label-free Mass Spectrometry technique / Manfredi, Marcello; Martinotti, Simona; Patrone, Mauro; Sassi, MARIA PAOLA; Ranzato, Elia; Marengo, Emilio. - (2015), pp. 526-530. [10.1109/MeMeA.2015.7145260]

*Availability:*

This version is available at: 11696/52567 since: 2016-04-11T16:51:17Z

*Publisher:*

*Published*

DOI:10.1109/MeMeA.2015.7145260

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# *Targeted Quantitation of HMGB1 Protein by label-free Mass Spectrometry Technique*

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**Abstract** — Mass spectrometry (MS)-based methods have become popular in recent years for the quantitation of biomolecules, especially peptides and proteins. High Mobility Group Box-1 protein (HMGB1) is a nuclear DNA-binding protein that resides inside the nucleus and can be released to the extracellular space under specific conditions. HMGB1 has been reported as circulating mechanistic indicators of cell death mode in animal models and in clinical studies. There is accumulating evidence that HMGB1 contributes to the pathogenesis of inflammatory and autoimmune diseases. Moreover, HMGB1 has been found to play an important role in tumor development, growth, and spread. Circulating HMGB1 level could be a useful and specific marker for evaluating different disease status and predicting prognosis in patients. The label-free quantitative comparison without any standard reference or normalization of the target peptide signal is the simplest Liquid Chromatographic-MS (LC-MS) method for the quantitation of a protein. The aim of the research was the development of an analytical method for the quantitation of HMGB1 by a label-free Mass Spectrometry technique. In this paper are presented the preliminary results: the quantitation of HMGB1 was assessed in a cell lysate sample through the use of the standard concentration curve of purified HMGB1 protein.

**Keywords**— *Targeted proteomics; shotgun analysis; HMGB1 quantification.*

## I. INTRODUCTION

The identification and quantification of proteins is widely used in biochemistry. These analyses are mainly performed in

order to measure changes in protein expression and post-translational modifications (PTMs) in biological samples. The most diffuse proteomic approaches are 2D electrophoresis and liquid chromatography (LC-MS): these methods, which includes a number of well-established options like MS-based methods, coupled to protein (or peptide) separation and bioinformatics analysis allow the achievement of protein identification and quantification [1]. The identification of protein PTMs is very relevant because they can cause significant changes of the protein's physical and chemical properties, activity, localization and stability [2]. Biomarkers discovery in proteomics is often performed by the use of high-throughput techniques providing a great number of candidates among which the true biomarkers have to be searched for [3].

There are several methodologies for carrying out the quantitation of proteins using both stable isotope labelling or label-free workflows [4-8]. Peptides with sequences unique to the target protein are usually selected as its surrogates. Peak areas of multiple reaction monitoring (MRM) transitions, which are the signal created by the peptide fragmentation, are integrated as measures of peptide abundance and are used as basis for quantitative comparison [9]. During last years, several software tools for design and analysis of large scale MRM proteomics datasets were developed [10].

High mobility group box-1 (HMGB1) is a highly conserved eukaryotic non-histone chromosomal protein of 215 amino acids. HMGB1 has attracted great interest as a prototype for a dual-function molecule. Indeed, HMGB1 has highly diverse

biological properties that extend from DNA binding to transcriptional regulation to chromatin architecture and to immune activation. Upon stimulation, HMGB1 undergoes translocation from the nucleus to the cytoplasm, and it is then secreted in most cells [11].

In this work, we developed an analytical method for the quantification of HMGB1 proteins using a typical shotgun approach: the development of the method includes the sample digestion with trypsin and the identification and quantification of HMGB1 specific peptides in a complex matrix.

## II. EXPERIMENTAL

### A. Sample preparation

HMGB1 purified protein was used to generate standard curves in a complex matrix such a conditioned medium of a cancer cell line (sarcomatous malignant mesothelioma MM98 cell line) [12] spanning from 300 attomoles to 6 femtomoles with four concentration levels (7.5, 15, 75 and 150 pg/uL). Linear equations for the calibration curves were determined by

non-weighted linear regression.

The eukaryotic recombinant HMGB1 protein was produced by using the Baculovirus system, and it was purified as previously described [13]. MM98 cells were seeded in 100 mm diameter plastic dishes with F-10 Ham without serum (Sigma Aldrich, Milan, Italy). The conditioned medium (CM) was routinely collected, 0.2  $\mu$ M were filtered, and maintained at -20°C. The protein amount of CM, after centrifugation at 300,000 rpm for 2 h, was determined with Bicinchoninic Acid (BCA) protein assay (Sigma Aldrich, Milan, Italy)[12]. After quantification, the sample was alkylated and reduced and then overnight digested with trypsin (Roche, Basel, Swiss).

### B. Data acquisition

The data acquisition was performed with a Triple TOF 5600+ system (AB SCIEX, Concord, ON) equipped with a DuoSpray™ Ion Source. The instrument was coupled to an Eksigent micro-LC system (Eksigent, Dublin, CA) with a C18 reverse phase column (HALOFused C18, 100 x 0.5 mm, Eksigent). Mobile phase A (0.1% (v/v) formic acid in water) and B (0.1% (v/v) formic acid in acetonitrile) were used to

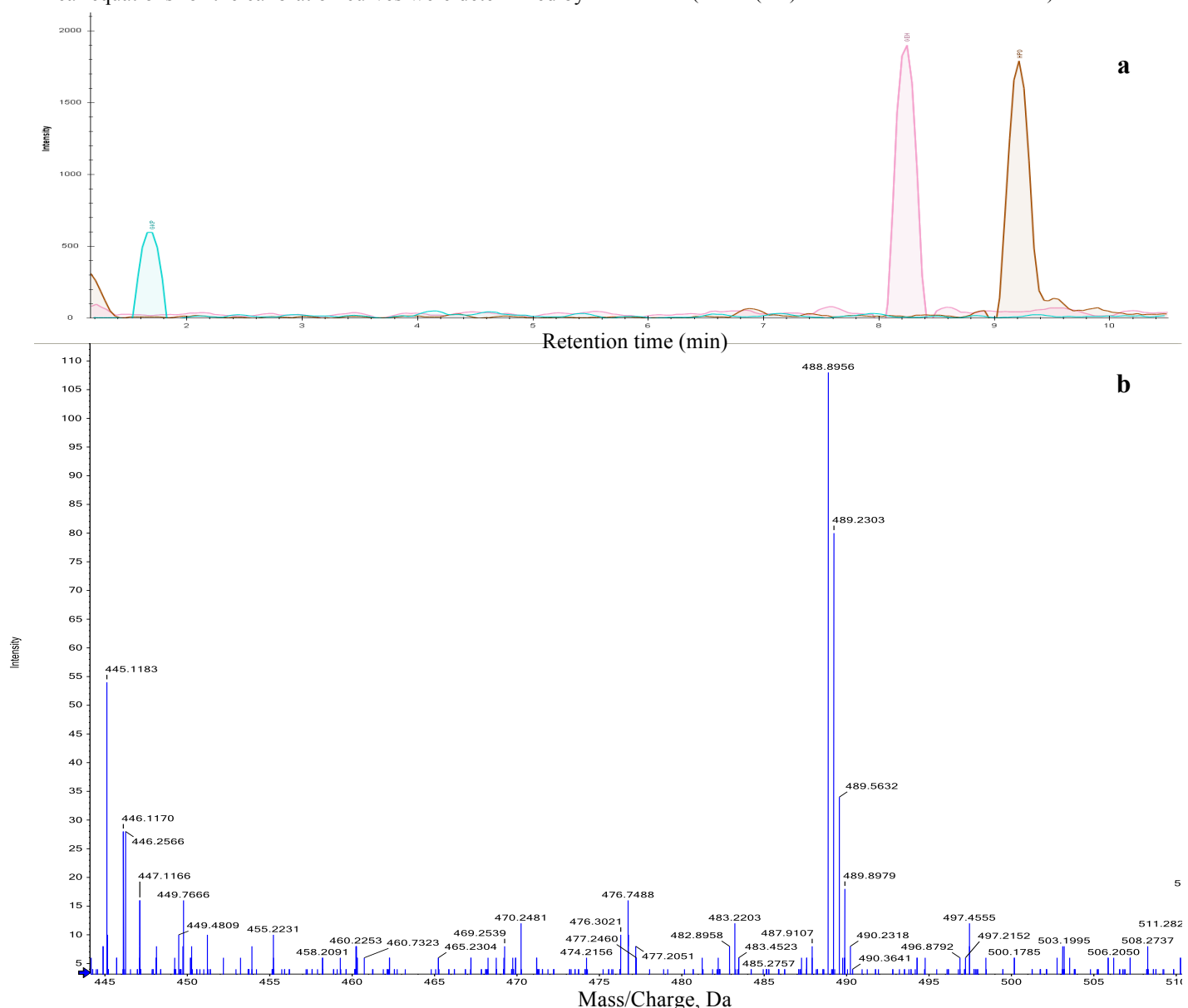


Fig. 1: (a) Chromatogram and peaks intensities traces for three HMGB1 peptides monitored for quantitative analysis. The first peak is from the peptide GKPDAAK, the second is from GEHPGLSIGDVAK and the third one is from the peptide HPDASVNFSEFSK which has been used for the quantification; (b) MS spectra of the peptide HPDASVNFSEFSK with 488.8956 m/z.

create the separation gradient for eluting peptides in the column at an increasing concentration of solvent B from 2% to 40 % in 30 minutes. Data were first acquired in the IDA mode. Peptide profiling was performed using a mass range of 350–1250 Da, followed by a MS/MS product ion scan from 100 to 1500 Da with the abundance threshold set at more than 120 cps. The accumulation time for ions was set at 50 ms. Then, samples were acquired with Multiple Reaction Monitoring (MRM) acquisition mode that provides a wide dynamic range, high sensitivity and low measurement variation. The ion source parameters in positive turbo ionspray mode were as follows: curtain gas 25 psi, GAS1 25 psi, and GAS2 20 psi, ionspray voltage 5000 V and source temperature 450°C. Collision energy for each monitored transitions were automatically calculated with Skyline software and exported to Analyst Software for the data acquisition. The collision energies (CE) and declustering potential (DP) used for the analysis of the three selected peptides were: CE 22.5 and DP 66.8 for HPDASVNFSEFSK, CE 31 and DP 77.8 for GEHPGLSIGDVAK and CE 17.3 and DP 56.2 for GKPDAAK.

The Triple TOF combines high resolving power (30k) and mass accuracy (<3ppm) with high MS/MS spectral acquisition rates (20Hz) that enables a powerful LC-MS/MS discovery and quantification platform.

### C. Data analysis

Data analysis was performed using Skyline 1.2 (MacCoss Lab Software) and Multiquant™ (ABSciex, Concord, ON, Canada) using peptide ion peak areas for quantification and PeakView™ 1.2.0.3 software (ABSciex, Concord, ON, Canada).

## III. RESULTS AND DISCUSSION

The concentration curve with injections from the lowest to the highest spike concentrations was acquired and three replicates of each point were performed to assess the reproducibility of the analysis. The curve for the peptide HPDASVNFSEFSK demonstrates an excellent linear response with good replicate reproducibility and an  $R^2$  of 0.9951. Coefficients of variation (CV) were calculated at each concentration point across the three replicates: all the CV were under 20% (19.75 %, 19.51%, 10.04% and 7.3% from the lowest to the highest concentration).

The limit of detection LOD was 2 pg/ $\mu$ L and has been calculated as the concentration of the analyte that gives a signal (peak area) equal to the average background ( $S_{\text{blank}}$ ) plus three times the standard deviation of the blank ( $\text{LOD} = S_{\text{blank}} + 3S_{\text{blank}}$ ), while the limit of quantification LOQ is given as  $\text{LOQ} = S_{\text{blank}} + 10 \cdot S_{\text{blank}}$  and was calculated as 6 pg/ $\mu$ L [14].

For the development of the method the free software Skyline was used. Figure 1 shows the chromatogram and peaks intensities traces (a) for three HMGB1 peptides monitored for

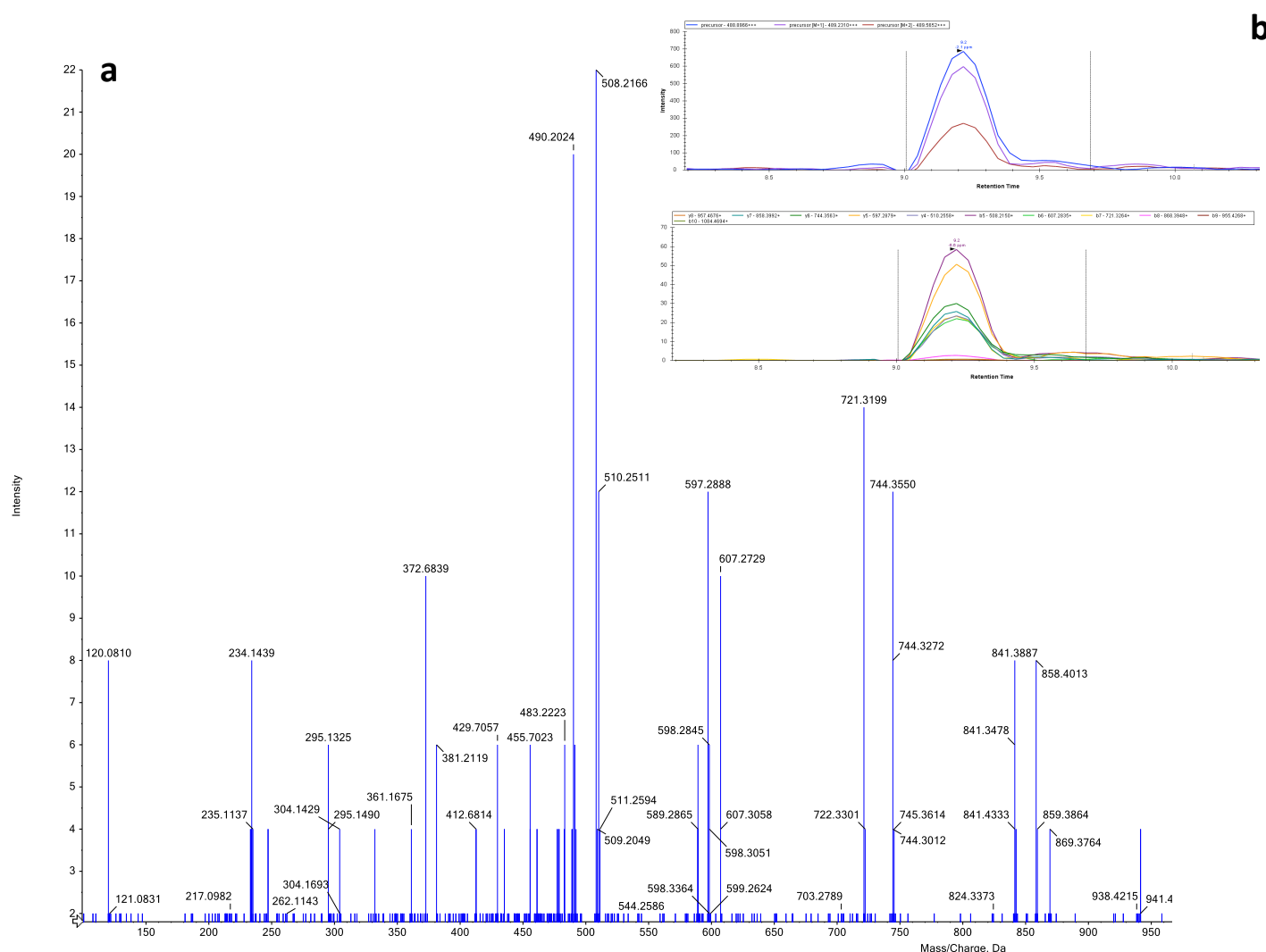


Fig. 2: (a) MS/MS spectra and (b) SRM traces for the selected transitions of the peptide HPDASVNFSEFSK

quantitative analysis. The first peak is from the peptide GKPDAAK, the second is from GEHPGLSIGDVAK and the third one is from the peptide HPDASVNFSEFSK which has been used for the quantification. MS spectra of the peptide HPDASVNFSEFSK with 488.8956 m/z is shown in figure 1b. The selected peptide uniquely identify the targeted protein: parallel acquisition of several transitions are used to validate the analysis since at the time of peptide elution, such transitions yield a perfect set of “co-eluting” intensity peaks because derived from the same peptide. Moreover, the monitoring of multiple transitions per peptide improves the quantitative robustness of the assay. Figure 2 shows the MS/MS spectra and the SRM traces for the selected transitions of the peptide HPDASVNFSEFSK that was used for the quantitation.

interfering with the detection and precluding the quantification of HMGB1 in biological matrices. The method developed in this work is able to quantify HMGB1 proteins using a typical shotgun approach: using a specific peptide of HMGB1 its quantification at low concentration could be performed. The developed technique could be useful in an environment where rapid turnaround of protein samples is needed, as well as analytical precision. Aside from improving the efficiency and speed of the experiments, future efforts will involve performing a more complete validation training to demonstrate the full bio analytical viability of this approach in different complex matrices such as cell lysates and biological fluids and the detection's feasibility off different HMGB1 post-translational modifications.

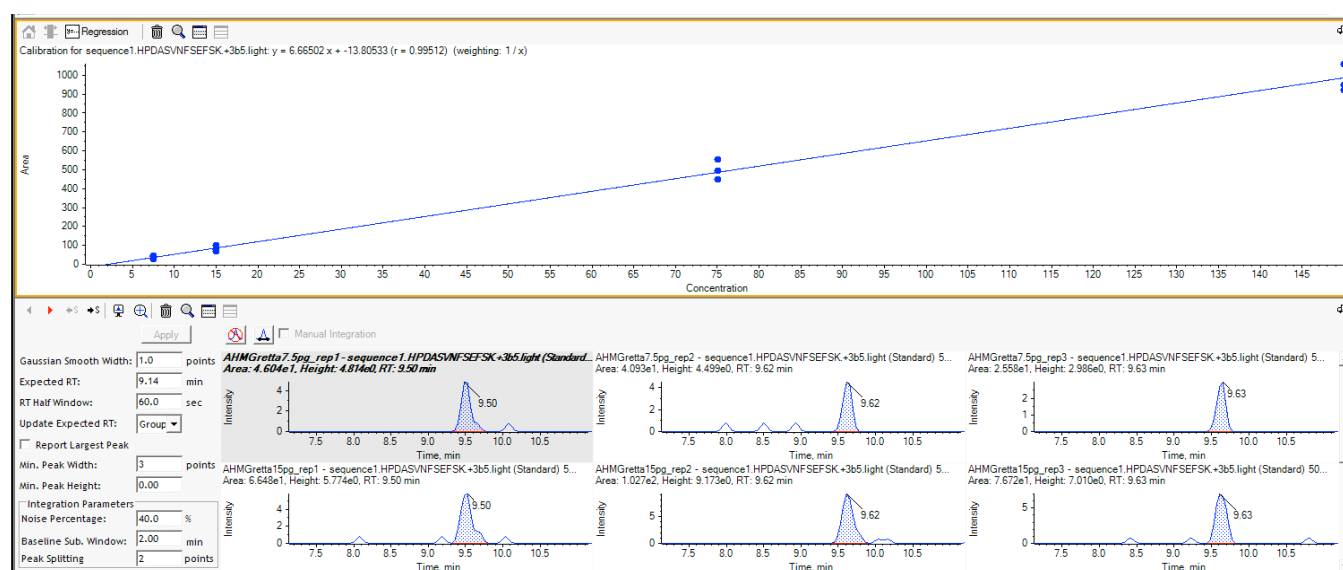


Fig. 3: Calibration curve for the peptide HPDASVNFSEFSK of HMGB1 protein and peaks area used to build the curve.

The calibration curve for the selected peptide used for quantification of HMGB1 protein is represented in figure 3. The calibration curve can be calculated using the sum of the area of multiple transitions or exclusively based on MS1 filtered precursor peak area. Using Multiquant software the absolute quantification of HMGB1 can be carried out in a biological sample by comparing the MRM response to the standard concentration curve.

#### IV. CONCLUSIONS

Many data indicate that HMGB1 might be a diagnostic marker, a potent mediator and a therapeutic target in several inflammatory, autoimmune and malignant diseases. However, so far, the detection of HMGB1 is performed by time-consuming, semi-quantitative Western blotting. Although ELISA approach has been proposed as a convenient way to quantify HMGB1 in biological fluids, various molecules have been shown to form complexes with HMGB1 and may interfere with HMGB1 detection by this technique. So, the sensitivity of these methods are limited by several factors

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