A high throughput MALDI-TOF mass spectrometry method for quantification of hepcidin in human urine

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S2 Supplemental Table 1.
Assay validation figures of merit.

S3 Supplemental Figure 1.
Preanalytical factors. A. Sequence of urine treatment prior to MALDI-TOF analysis, B. Consecutive freeze/thaw treatments, C. Spectra following third day of freeze/thaw treatment, D. Influence of I.S. on HepC peak intensity.

S4 Supplemental Figure 2.
Representative set of spectra from urine cross validation. Inset: magnified view showing isotopic peak series for HepC-25 and the internal standard

S5-7 Supplemental Figure 3.
Representative set of spectra demonstrating methionine oxidation and the concurrent appearance of metastable decay peaks originating from neutral loss of the sulfoxide ion (CH_3SOH).

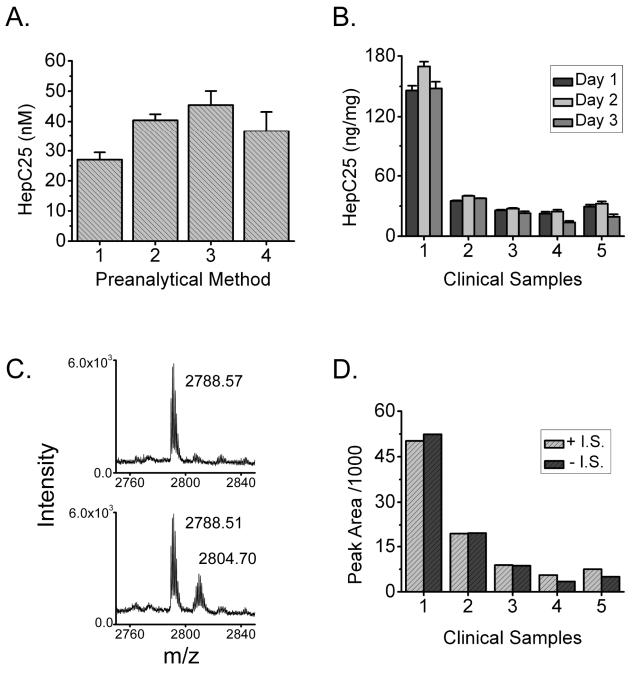
Table 1.

Linearity, accuracy, and precision measurements for assay validation.

Linearity				
Concentration (nM)	300	100	30	10
Mean (nM) n=6	311.42	97.22	31.38	10.10
Accuracy (% Recovery) n=6	103.81	97.22	104.6	101
Precision (% CV) n=6	5.30	5.21	10.05	13.17
Dilution Linearity	Sample 1	Dilution 1	Dilution 2	Dilution 3
Concentration (nM)	101.12	33.71	16.85	8.43
Mean (nM)	94.50	34.11	16.80	7.59
Accuracy (% Recovery)	93.45	101.19	99.70	90.04
Accuracy				
Sample Recovery	Sample 1	Recovery 1	Recovery 2	Recovery 3
Mean (nM)	40.90	50.96	106.45	334.94
HepC-25 Added (nM)		10	60	300
Recovered (nM)		10.06	65.55	294.04
Recovery (%)		101	109	98
Analyte Interference	Sample 1	Interference 1	Interference 2	
Mean (nM)	101.12	114.74	108.89	
NaCl Added (M)	0	0.25	1.0	
Interference (nM)		13.63	7.78	
Precision				
Inter-spot/Inter-day	Measurements	Sample 1	Sample 2	Sample 3
Day 1	Mean (nM) Precision (%CV) n	106.05 5.75 6	25.33 11.28 6	10.30 20.47 4
Day 2	Mean (nM) Precision (%CV) n	110.77 7.38 6	30.23 11.41 6	11.97 10.04 6
Day 3	Mean (nM) Precision (%CV) n	101.79 9.43 6	29.76 12.42 6	11.78 23.63 6
Total	Mean (nM) Precision (%CV) n	106.20 7.92 18	28.44 13.65 18	11.48 18.38 16

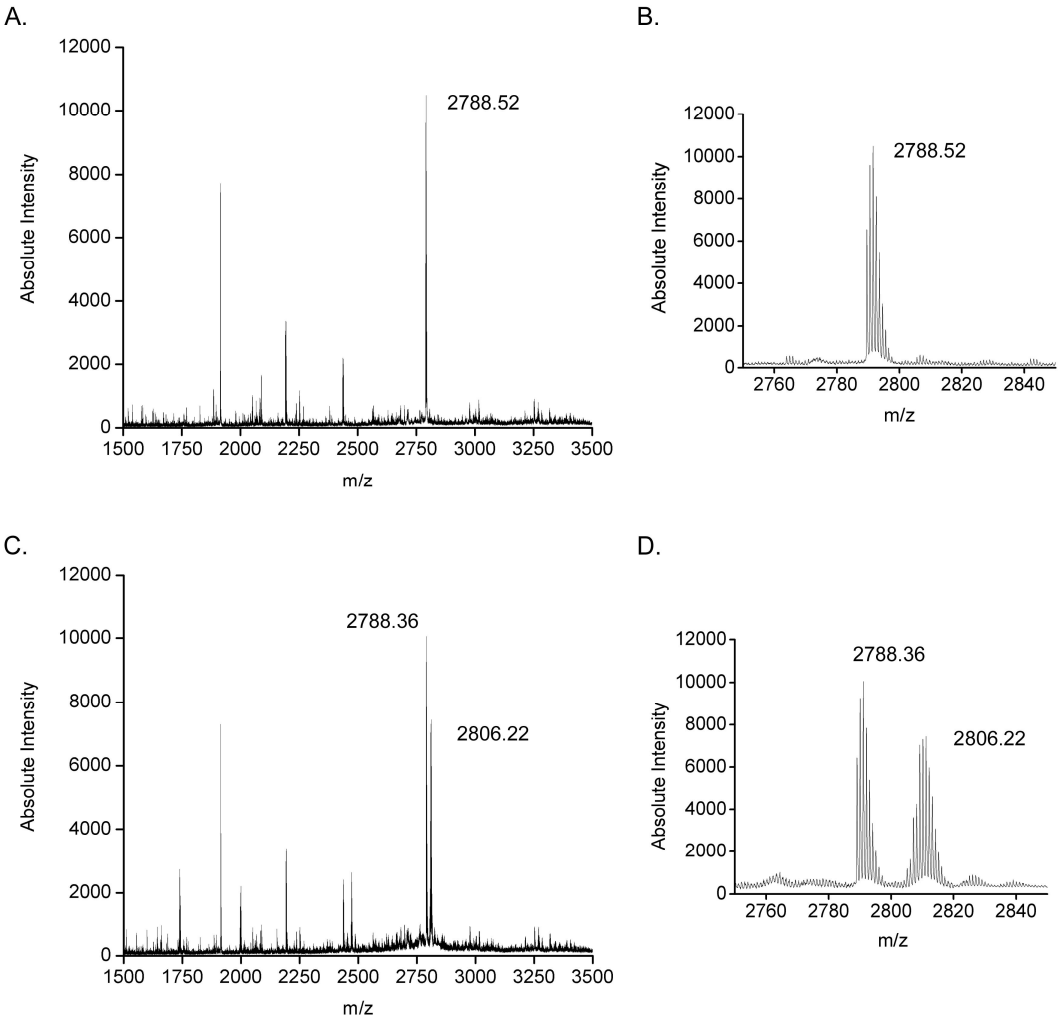
Supplemental Figure 1.

Assessment of pre-analytical factors impacting assay performance. A. The sequence of urine treatment prior to direct on-chip enrichment and MALDI-TOF analysis. Method 1 – centrifugation, acidification, internal standard addition, Method 2 – acidification, centrifugation, I.S. addition, Method 3 – I.S. addition, acidification, centrifugation, Method 4 – no centrifugation step. Shown are samples from a single urine sample analyzed in duplicate, errors bars are S.D. B. The effects of successive freeze/thaw cycles on HepC-25 measurement assessed using five urine samples tested over a three day period, values are normalized for urine creatinine. C. Representative spectra showing a sample with and without internal standard following the third day of freeze/thaw treatment. D. Assessment of the potential influence of internal standard on HepC-25 ion suppression. Five urine samples were analyzed with and without added internal standard and peak area of HepC-25 was plotted.



Supplemental Figure 2.

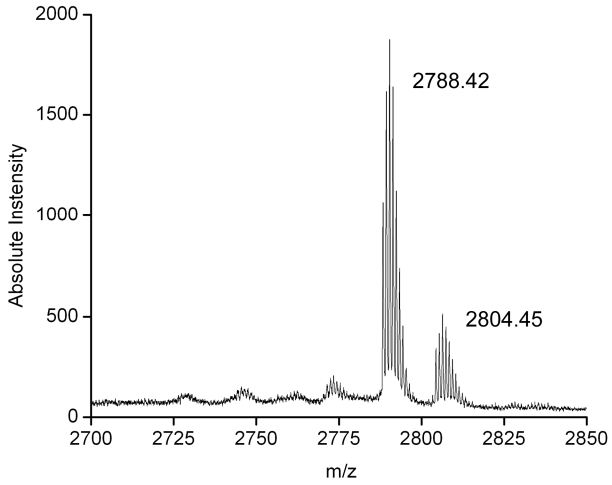
Representative spectra from urine sample cross validation. A. Spectrum of sample in the absence of I.S. B. Magnified section of the spectrum detailing the HepC-25 peak isotope series; note the absence of methionine oxidation (+16 m/z). C. Spectrum of sample with added I.S. (100 nM). D. Magnified section of the spectrum detailing the HepC-25 and HepC-25($Lys^{15}N_2{}^{13}C_6)_2$ peak isotope series.



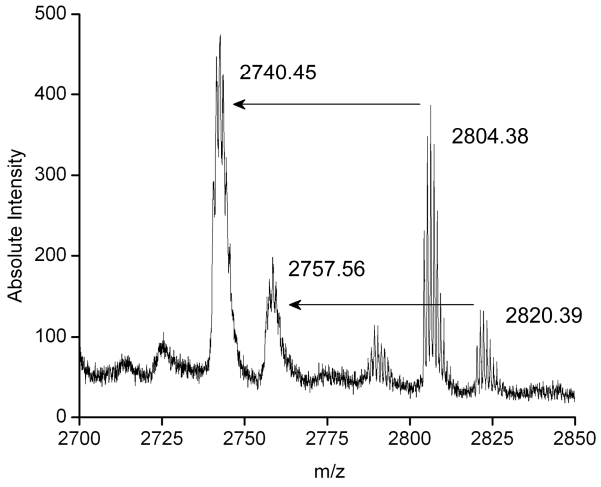
Supplemental Figure 3.

Previous reports have noted potential assay interference caused by hepcidin methionine oxidation, likely arising from handling and manipulation or urine under oxidative conditions during the enrichment phase of hepcidin analysis [1-3]. All described methods to date involve incubation/agitation (typically ≥30 min at r.t.) of urine at neutral pH with solid phase binding substrate (WCX or IMAC SELDI or IEX resin) prior to elution and analysis. We observed nominal oxidation in our experiments as the described method involves minimal handling (time and sample transfers) and thus minimal sample exposure to oxidative conditions (see text and Supplemental Figures 1, 2). Nonetheless, in order to investigate the effects of potential oxidation, we incubated a normal urine sample (Figure 3 panel a) with 0.6% H_2O_2 to induce oxidation prior to WCX enrichment and analysis (Figure 3 panel B). Interestingly, we observed the appearance of metastable decay peaks (Δ64 m/z (peaks 2740.45 and 2757.56) in the oxidized sample representing an apparent loss of the sulfoxide (CH_3SOH) from oxidized methionine, which arises from post source decay of oxidized methionine in the mass spectrometer. Although we anticipate no confounding issues regarding oxidation in our studies, the observation of the intense metastable decay ions is useful as a diagnostic (or correction factor) for methods involving extended sample handling and enrichment under oxidative conditions, or in the assessment of clinical samples that may have been handled under less than ideal conditions.

A.



B.



(1) Swinkels, D. W.; Girelli, D.; Laarakkers, C.; Kroot, J.; Campostrini, N.; Kemna, E. H.; Tjalsma, H. *PLoS ONE* **2008**, *3*, e2706.

(2) Ward, D. G.; Roberts, K.; Stonelake, P.; Goon, P.; Zampronio, C. G.; Martin, A.; Johnson, P. J.; Iqbal, T.; Tselepis, C. *Proteome Sci* **2008**, *6*, 28.

(3) Cohen, S. L. *Anal Chem* **2006**, *78*, 4352-4362.