Sample extraction.
All six samples were extracted simultaneously using mixed mode SPE cartridges. The proteins were eluted in solvent containing 80% ACN and evaporated overnight. Total extraction time was 30 minutes.

Reduction, alkylation and digestion.
Samples were reconstituted into 50 mM ammonium bicarbonate, with 10 mM DTT, and incubated at 60 °C for 60 minutes. Iodoacetamide was added to a final concentration of 20 mM and the samples incubated at RT for 30 mins. 1 µg of Trypsin was added and incubated overnight at 37 °C. Samples were brought up to a total formic acid concentration of 0.1% v/v before analysis. Total sample volume for analysis was 80 µL.

LC-MS/MS analysis.
Peptide MS/MS spectra on the ABRF website were used to identify suitable MRMs for analysis on a 5500 QTrap, however only hCG and PSA peptides were targeted. LC-MS/MS analysis was performed on a Dionex Ultimate 3000 at 500 µL/min using a 2.1 x 100mm C_{18} HPLC column. Gradient program involved starting at 10% ACN, rising to 35% over 3 minutes, and total method time was 5 minutes. Sample (5 µL of the 80 µL) was injected onto the column, and total analysis time for all six samples totalled 30 minutes. Peak areas from the peptide were calculated using Analyst 1.5. Figures 1 and 2 display MRM chromatograms for PSA and hCG respectively.

Relative quantitation of PSA and hCG.
Peak areas for each transition were expressed as a ratio to an albumin peptide peak, and relative levels of hCG and PSA in each sample were calculated. Paired samples were identified as A=E, B=F and C=D for both PSA and hCG.

Conclusions.
The paired samples were identified as A=E, B=F and C=D, for both proteins in the six samples. The relative levels of each protein in the duplicate samples gave % CV values between 2.6 and 19.6, which demonstrates the high reproducibility of the extraction and analysis technique. The SPE extraction and high flow rate analysis took ~4.5 hours total (not including overnight drying and digestion stages), demonstrating that relative concentrations of proteins in a clinical matrix can be achieved using high throughput extraction and LC-MS/MS analysis methodologies.

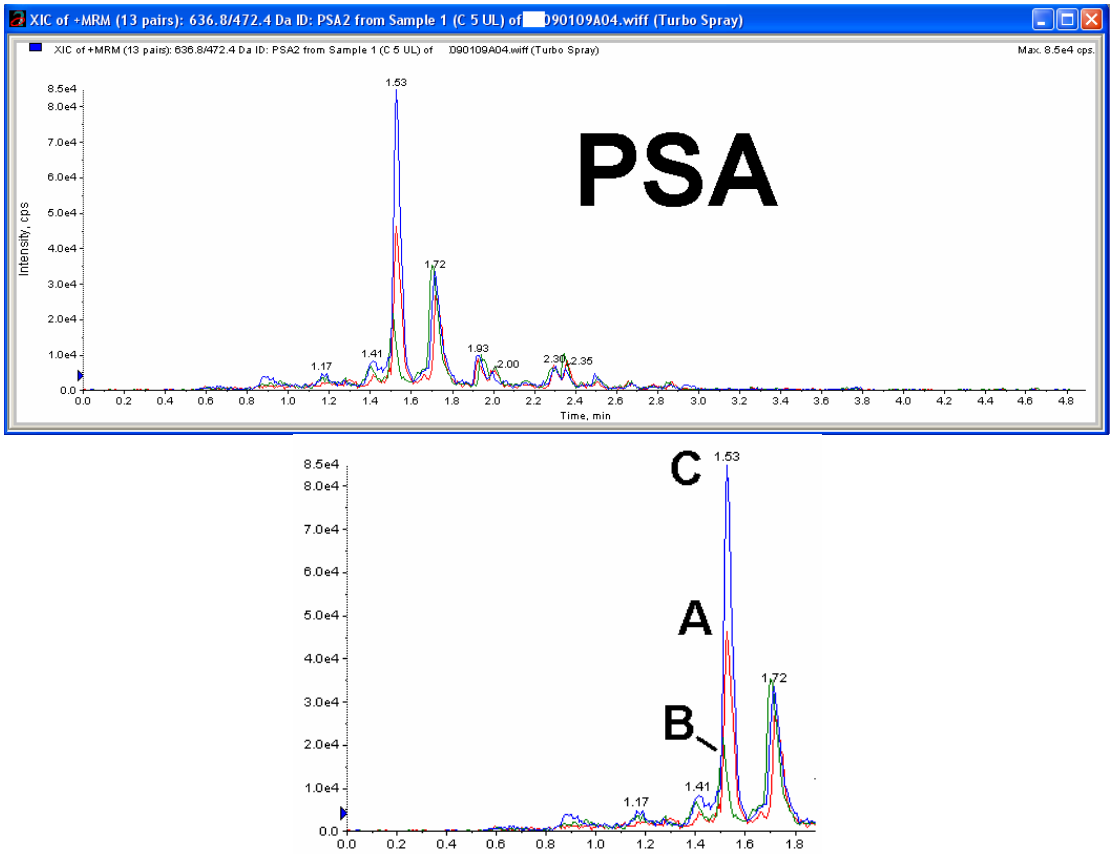

Figure 1 PSA MRM traces for samples A, B and C.

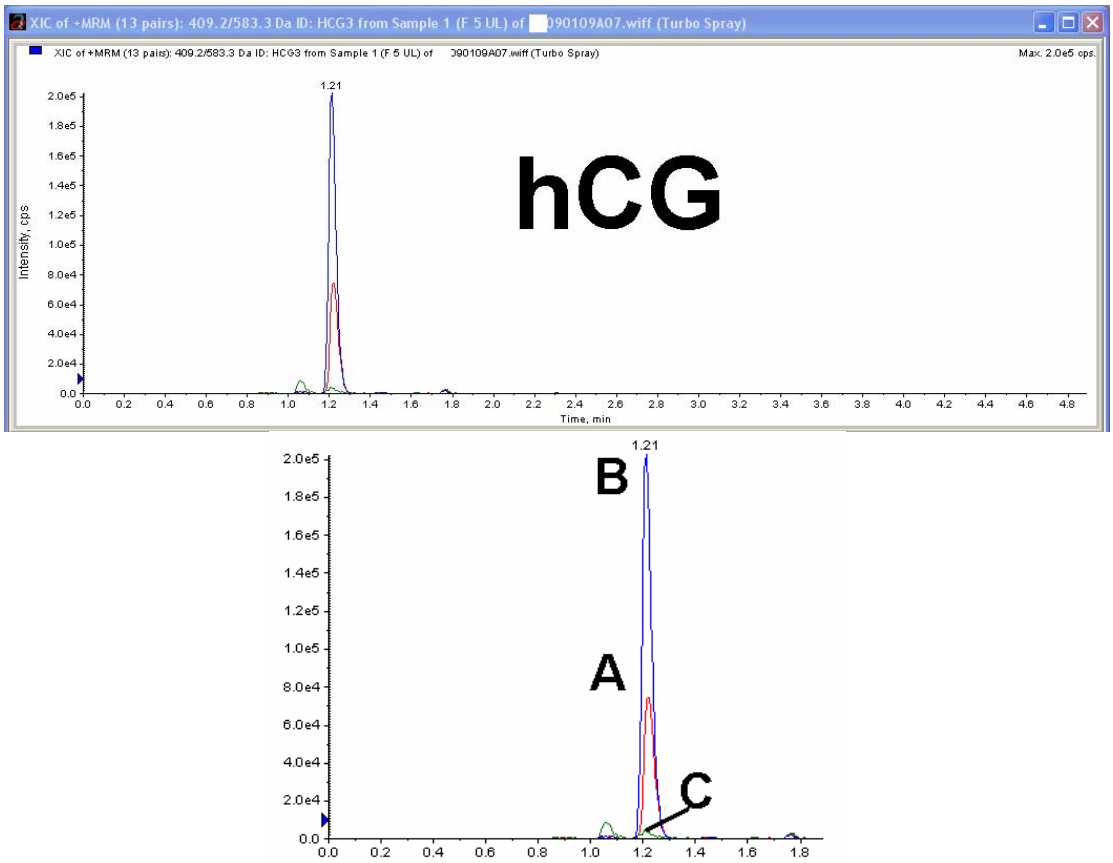

Figure 2. hCG MRM traces for samples A, B and C.