

## PROJECT OVERVIEW

- Phosphorylated proteins from a human endometrial cancer cell line were pre-concentrated using affinity chromatography.
- After tryptic digestion of the isolated proteins, TiO<sub>2</sub> nanocolumns were evaluated for their effectiveness in enriching phosphorylated peptides.
- C18 nanobore LC-MS/MS with use of the scan modes afforded by a QTRAP instrument enabled the detection of several phosphopeptides.

## INTRODUCTION

Protein phosphorylation in human cells is an important reversible process that is managed by kinases. These phosphorylation and dephosphorylation events control cell growth, division and intracellular signaling. De-regulation of protein phosphorylation, therefore, can be a sensitive indicator of cancer. Cancer affecting the body of the uterus is the fourth most common cancer in North American women<sup>1</sup> and the most common of all gynecologic malignancy.

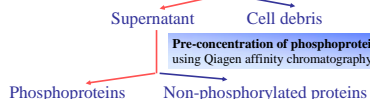
We aim to isolate phosphorylated proteins from human endometrial clinical cancer samples using a combination of separation and enrichment techniques and characterize them using mass spectrometry. Any modified protein whose levels differ from that found in the normal cell can be used as a potential biomarker, enabling both a rapid and accurate diagnosis of endometrial cancer (EmCa). Methods to achieve this have been developed using a human EmCa cell line.

## METHODS

### Sample Preparation and Work Up

EmCa Cells

Protease inhibitors, Na<sub>2</sub>VO<sub>4</sub> included in lysis buffer. Harvested and washed in Tris saline. Lysed 30 min, 4°C in 20 mM Tris pH 7.5, 150 mM NaCl, 1% NP40, 0.5% Na-deoxycholate, 1 mM EDTA



Detergent removal using Norgen's ProteoSpin Detergent Clean-up Kit

Detergent free sample, containing protein

Proteins reduced, alkylated and trypsinized

Sample containing proteolytic peptides

Desalting (C18 SPE column) – elution in 3 fractions (Acetonitrile step gradient)

(Phospho)Peptides ready for TiO<sub>2</sub> enrichment studies

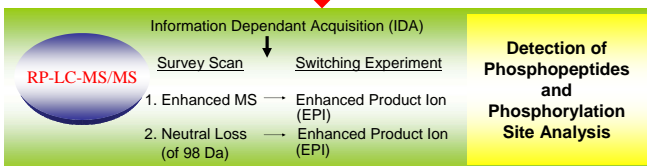
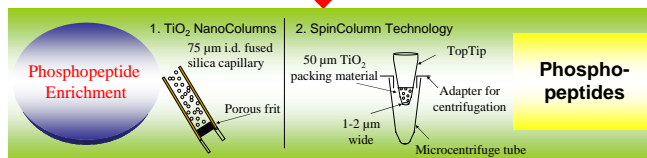
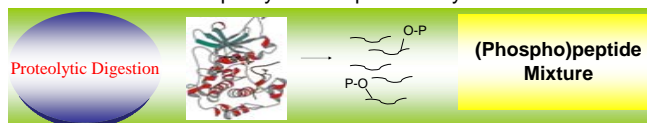
### Nanobore Liquid Chromatography – Tandem Mass Spectrometry (LC-MS/MS) Instrumentation and LC conditions

The LC system from Applied Biosystems/ MDS SCIEX, consisted of a nano LC autosampler; Eksigent Tempo nano MDLC pump and 0.1 x 150 mm Magic C18 5 μ 100 Å RP column (Michrom Bioresources). The LC system was interfaced with a 4000 Q TRAP mass spectrometer (Applied Biosystems/ MDS SCIEX, Concord, Canada)

### TiO<sub>2</sub> Enrichment of Phosphorylated Peptides

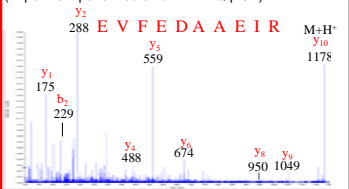
- Titansphere Nanocolumns – IntegraFrit columns with a 75 μm i.d. (New Objective) were packed with 3-μm titansphere particles; 100 Å, to a length of 15 cm
- The column was connected to the nano LC autosampler six-port injection valve, equipped with a 10 μL sample loop
- Eksigent Tempo nano MDLC pump was used to deliver 0.1% FA solvent
- Column conditioning, washing, elution and sample introduction were performed using a plug approach, each solution being supplied via the sample loop at a flow rate of 250 nL min<sup>-1</sup>
- Column conditioning: 2 x 0.1 M NH<sub>4</sub>HCO<sub>3</sub> (pH 9)
- Sample loading: Sample dissolved in 30% ACN, 2% FA
- Column washing: 30% ACN, 2% FA
- Column elution: 0.1 M NH<sub>4</sub>HCO<sub>3</sub> (pH 9)

### Phosphorylated Peptide Analysis



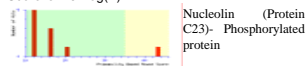
## RESULTS

Tandem MS of M+H<sup>+</sup> Peptide Ion at m/z 1178 Eluted from TiO<sub>2</sub> column (Experiment performed on a MALDI-QqTOF)



Results of NCBI Protein Database Search using Mascot Search Engine

Histogram showing probability Based Mowse Score using product ions of the precursor ion at m/z 1178 – Score is -10<sup>4</sup>Log(P)

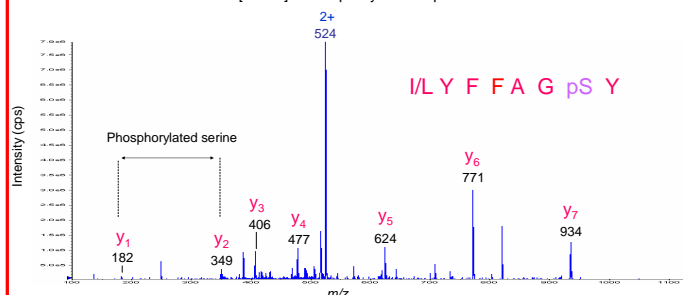


Observed Mr(exp)/Mr(calc) Score Peptide  
1178.60 1177.59 1177.60 46 K.EVFEDA A E I R.L  
Other peptides analysed by a similar approach gave significant hits for Neurotrophic tyrosine kinase receptor type 1 phosphorylated protein and Nucleolin C23 protein containing phosphorylated serines

Examples exist where non-phosphorylated peptides were observed in the elution fraction from the TiO<sub>2</sub> nanocolumn. Database hits for phosphorylated proteins however, suggests the effectiveness of the Qiagen pre-concentration of phosphoproteins.

Enhanced Product Ion Experiments Triggered by IDA after a Neutral Loss Survey Scan (NL 98Da) of TiO<sub>2</sub> Enriched Tryptic Peptides from Endometrial Cancer Cell Line using a 4000 Q TRAP LC/MS/MS System

Tandem MS of [M+2H]<sup>2+</sup> Phosphorylated Peptide Ion at m/z 524



- 30 EPI experiments were triggered after peptides underwent a NL for the elimination of the elements of phosphoric acid.
- 6 spectra contained ions that suggested the presence of phosphorylated serines/threonines or the loss of phosphate from these amino acid residues.
- The ions in the remaining spectra did not allow for full sequence determination as the loss of phosphoric acid upon CID frequently causes inadequate fragmentation of the peptide backbone.

## CONCLUSIONS

- Developed an effective sample preparation strategy based on
  - pre-concentration of phosphoproteins from EmCa cell line lysate using affinity chromatography
  - detergent removal using SiC column spin technology
  - TiO<sub>2</sub> phosphopeptide enrichment from a mixture of tryptic peptides
- 500 EPI experiments of tryptic peptides (from EmCa cell line) prior to TiO<sub>2</sub>, were narrowed down to 30 EPI experiments triggered by NL survey scans, enabling identification of 1 phosphorylation site and the tentative identification of 3 other phosphorylation sites.
- MALDI-QqTOF detected high abundant peptides and generated tandem data that, when used in database searches, enabled identification of phosphoproteins. On the other hand, the use of RP-LC (phospho)peptide separation followed by the effective use of the QTRAP scanning modes detected the less abundant phosphopeptides

## FUTURE DIRECTIONS

- Perform database searching using the data obtained from the LC-MS/MS
- Develop a NH<sub>4</sub>HCO<sub>3</sub> LC gradient method for elution of peptides from the TiO<sub>2</sub> nanocolumn incorporating DHB conditioning/washing and NH<sub>4</sub>OH elution steps that we have previously optimised when using TiO<sub>2</sub> TopTip SpinColumns
- Use other QTRAP tandem survey scans to detect phosphorylated peptides, including precursor ion experiments of m/z -79 (PO<sub>3</sub><sup>-</sup>) and +216 (pY immonium ion); MRM transitions

<sup>1</sup>Canadian Cancer Statistics 2005 – Compiled and produced by Canadian Cancer Society, National Cancer Institute of Canada, Statistics Canada, Provincial/Territorial Cancer Registries, Public Health Agency of Canada

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