# Proteomic Profiling of Complex Samples Using Multidimensional Protein and Peptide Separation Strategy and Accurate Mass Mass Spectrometry

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## Introduction

*Complex biological samples like body fluids, tissues, cell lysates, etc., are frequently used for the discovery of new disease markers, or studying protein expression patterns in biology. It is extremely challenging to identify and characterize low copy number proteins. The ability to characterize complex protein samples by mass spectrometry depends on the power of the separation techniques employed, prior to the MS analysis, in order to reduce sample complexity and increase sensitivity of the mass spectrometer.*

*Here we introduce a combination of affinity depletion, off-gel electrophoresis (OGE) and reversed-phase separation at the protein level to reduce sample complexity, followed by high throughput Chip based LC/MS analysis of serum samples from prostate*

## Methods

*Sample Preparation: Prostate cancer patient serum was obtained from Bioreclamation inc..*

*High Capacity Multiple Affinity Column: Agilent High Capacity Multiple Affinity Removal Column, 7.5mm i.d. x 100mm. 120ml serum was diluted 4x with the loading buffer A for each depletion run. Sample was loaded onto the column and the flow-through collected, pooled and concentrated.*

*OGE Fractionation: Off-Gel electrophoresis (Agilent 3100 OFFGEL fractionator) was performed using high resolution (pH 3-10) kit according to the OFFGEL Kit Guide. 1.5mg of depleted serum was reduced, alkylated and focused for 24h for in-solution fractionation.*

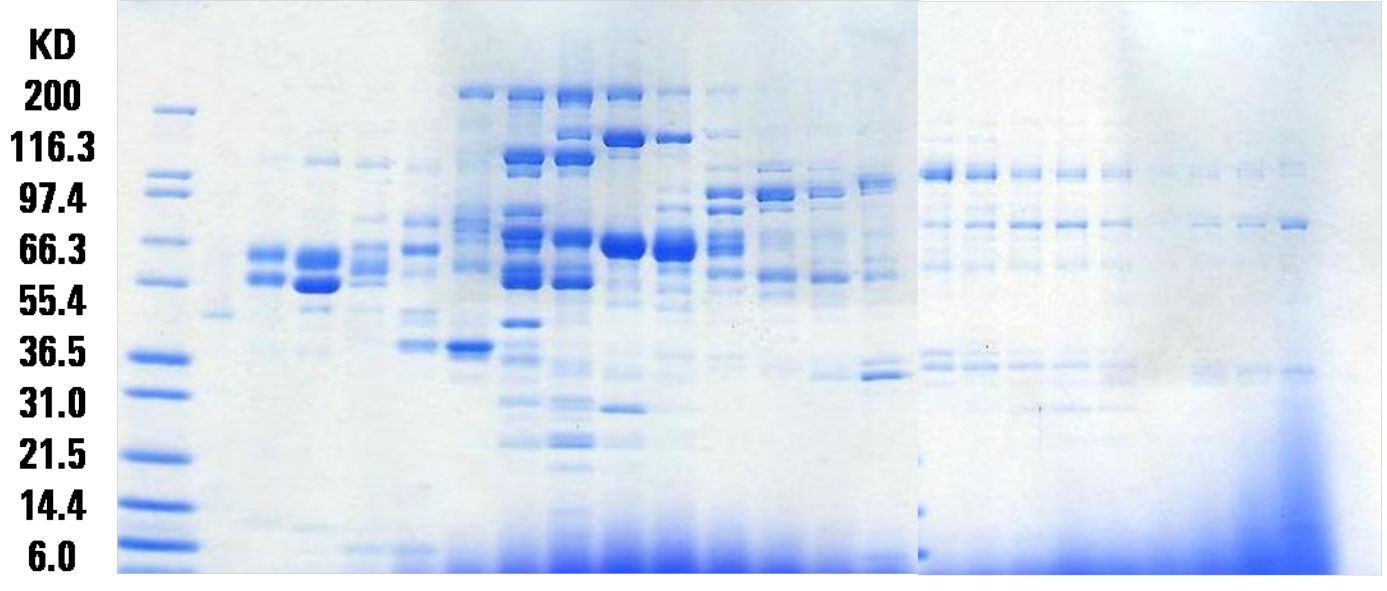
*Macroporous Reversed-phase (mRP) Separation: All the 24 OGE fractions were mRP separated using 5mm, 4.6 x 50mm column (Agilent) at 80°C using water (0.1% TFA)/acetonitrile (0.08% TFA) gradient. A total 20 mRP fractions were collected per each OGE fraction.*

*Trypsin digestion and LC/MS/MS Analysis: Dried mRP fractions were trypsin digested and peptides were analyzed on HPLC-Chip/6520 QTOF using 18 min method. 10% of the reconstituted peptides were directly injected onto a high capacity (HC) chip. The LC-MS/MS data was processed by Spectrum Mill software.*

## Experimental Workflow

# Results and Discussion

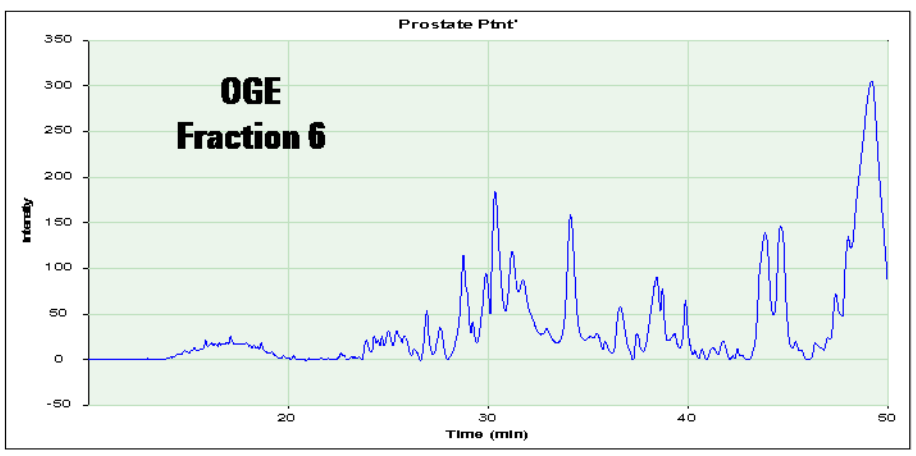
## SDS-PAGE of OGE Fractions

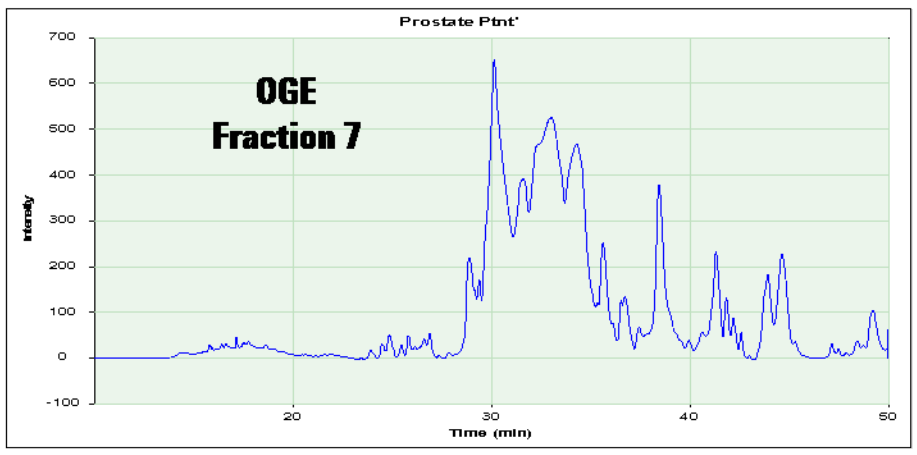



**Lane 1: Protein Marker**
**Lane 2-25: OGE fractions**

**SDS-PAGE gel image of depleted human serum OGE fractions. The completeness of removal of 13 targeted proteins by depletion column has been determined to be more than 99% by ELISA. 5µl sample from each of 24 OGE fractions were mixed with 5µl sample buffer and then loaded onto a 15 well SDS-PAGE (4-20%) gel.**

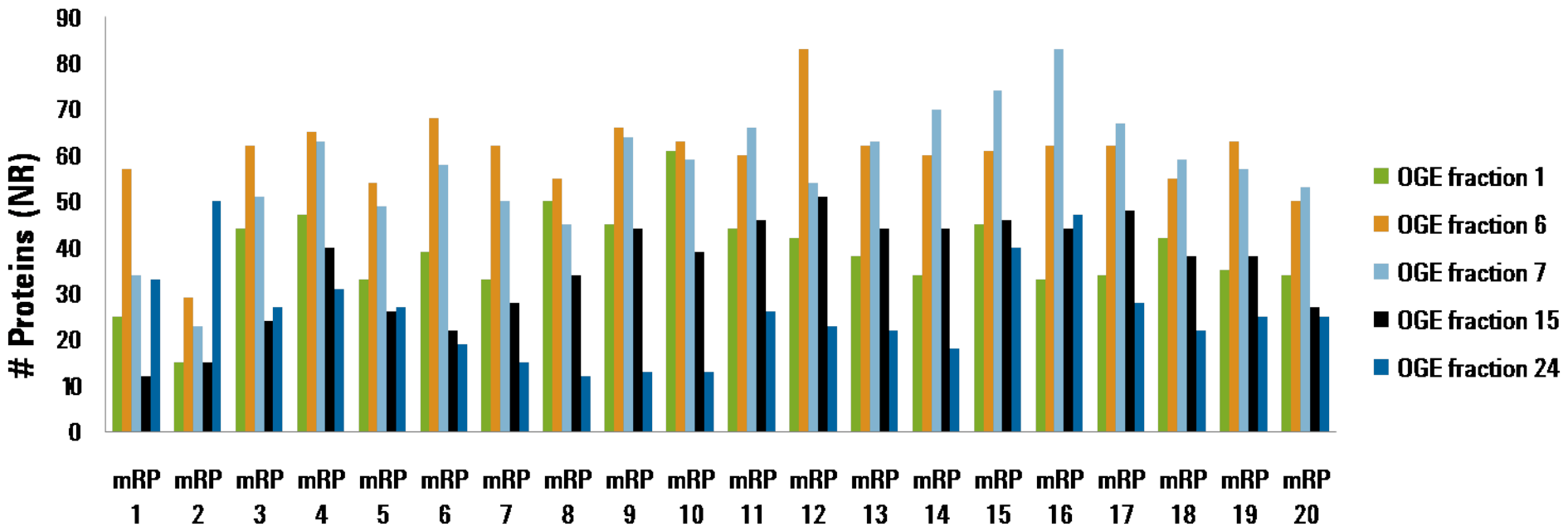
## Protein mRP Separation of OGE Fractions




**Each OGE protein fractions were further fractionated into 20 mRP fractions which were collected at 2-minute time slices from 8-48 min. A total of 480 fractions were collected for HPLC-chip/MS analysis**

## Distinct Proteins Found in Selected mRP Fractions

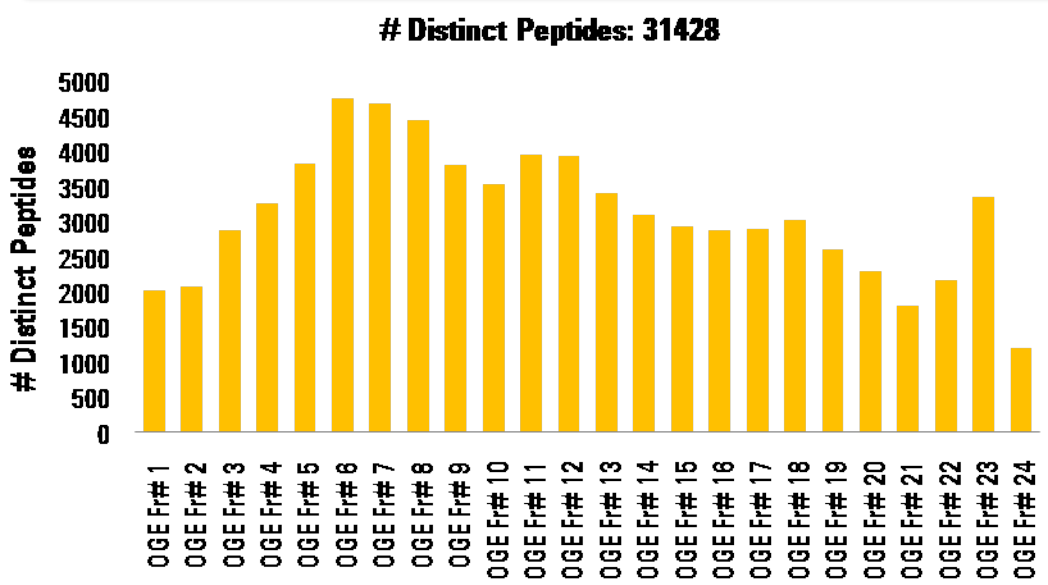



**Each bar represents the total number of proteins found in individual mRP fractions from selected OGE fractions. Total 20 mRP fractions were collected/OGE fraction, dried and trypsin digested. 30% of peptides from each mRP fractions were analysed on a HPLC chip QTOF mass spectrometer using 15 min gradient and MS/MS data was processed using Spectrum Mill software.**

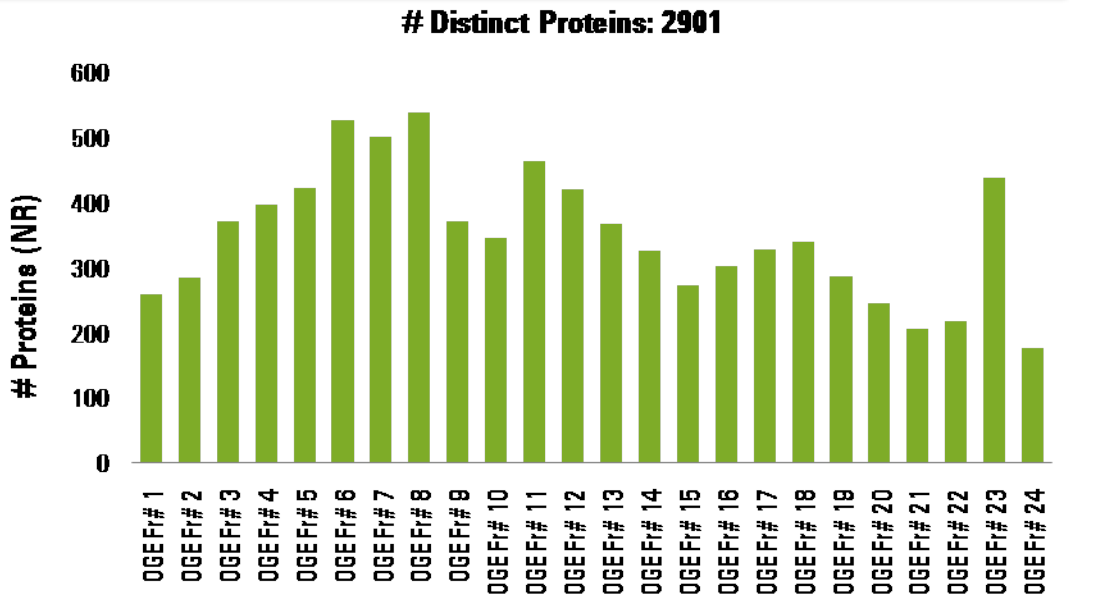
# Results and Discussion

## Distinct Peptides and Proteins Found in Individual OGE Fractions

| OGEFr # | OGE 01 | OGE 02 | OGE 03 | OGE 04 | OGE 05 | OGE 0 6 | OGE 07 | OGE 08 | OGE 09 | OGE 10 | OGE 11 | OGE 12 | OGE 13 | OGE 14 | OGE 15 | OGE 16 | OGE 17 | OGE 18 | OGE 19 | OGE 20 | OGE 21 | OGE 22 | OGE 23 | OGE 24 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| % FDR | 0 | 0 | 3.23 | 3.02 | 0.47 | 1.52 | 1.2 | 1.49 | 0.54 | 2.32 | 1.72 | 2.86 | 3.27 | 2.45 | 0 | 0.66 | 0.61 | 1.76 | 0 | 0 | 0.98 | 0.92 | 2.28 | 1.13 |




**Each bar represents the total number of unique peptides found in individual OGE fractions which are further fractionated to 20 mRP fractions. Trypsin digested mRP fractions were analysed on HPLC-Chip QTOF Mass Spectrometer using HC Chip. MS/MS data was searched against human IPI database using spectrum mill bioinformatics software.**




**Each bar represents the total number of proteins found in individual OGE fractions which are further fractionated to 20 mRP fractions. Spectrum Mill autovalidation yielded about 2900 unique human proteins with >2 peptides/protein (<2.5% FDR) in total of 480 mRP fractions.**

## List of Identified Low Abundant Proteins

| Protein Names | Number of Unique Peptides | Spectrum Mill Score |
|---|---|---|
| Isoform A of Proteoglycan 4 | 44 | 418.42 |
| Vascular cell adhesion protein 1 isoform a | 25 | 274.59 |
| Platelet factor 4 | 22 | 274.38 |
| Insulin-like growth factor-binding protein 4 | 17 | 228.37 |
| Hepatocyte growth factor-like Protein | 11 | 170.66 |
| Insulin-like growth factor 2 isoform 2 | 13 | 162.81 |
| Intercellular adhesion molecule 1 | 18 | 162.75 |
| Insulin-like growth factor IA | 9 | 135.72 |
| Vascular endothelial growth factor receptor 1 | 25 | 128.02 |
| IL16 Isoform 1 of Pro-interleukin-16 | 25 | 124.95 |

| Protein Names | Number of Unique Peptides | Spectrum Mill Score |
|---|---|---|
| Insulin-like growth factor binding protein 7 | 9 | 110.09 |
| Cathepsin D | 11 | 107.94 |
| Macrophage colony-stimulating factor 1 | 13 | 85.85 |
| Metalloproteinase inhibitor 2 | 7 | 83.85 |
| Secretoglobin, family 1A (uteroglobin) | 6 | 77.42 |
| Intercellular adhesion molecule 2 | 5 | 45.33 |
| IL20 Interleukin 20 short form | 6 | 34.29 |
| Myoglobin, isoform CRA_a | 4 | 41.39 |
| Troponin C | 4 | 28.25 |
| Pro-platelet basic protein precursor | 2 | 22.14 |

**Table 1: List of low abundant proteins (pg to ng/ml) identified in depleted prostate cancer patient serum including known cytokines and growth factors with >2 Peptides. Only proteins observed with two or more peptides were considered as confident identification. The number of unique peptides for a given protein is the combined results from all the mRP fractions.**

# Conclusions

1. **High capacity affinity removal column, off-gel electrophoresis and high temperature reverse phase separation techniques greatly reduced the sample complexity.**
2. **HPLC-Chip QTOF technology allowed to do high throughput LC-MS/MS analysis and entire analysis was completed in less than a week.**
3. **Depletion of 14 high abundant plasma proteins and extensive protein fractionation significantly increased the detection limit and allowed us to identify many low abundant (ng/ml) proteins, including known cytokines and growth factors.**
4. **This workflow may provide utility to identify low abundant proteins critical to pathological events and pharmacologic intervention from a variety of sample sources.**