

## A proteomic strategy to analyze secreted factors from cell culture

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### Introduction

The discovery of secreted factors from cells has been a major focus of proteomic analysis for a number of years, driven both by biomarker discovery as well as understanding how cells effect their local environment. Generally, cell culture supernatant is analyzed free of serum supplement to avoid the presence of inherent high abundance proteins. The problem is that cultured cells are stressed in the serum free environment resulting in atypical growth. This study describes the use of a commercially available serum reagent to supplement normal cell culture growth. This supplement, in comparison to normal serum supplements, contains a limited number of proteins with bovine serum albumin (BSA) being the only high abundance one (Table 1).<sup>1</sup> We compare and contrast different biochemical and chromatographic approaches to selectively remove the BSA from the supernatant, allowing for LC-MS/MS identification of low abundance, secreted factors. Our system investigated here is media that has been conditioned by mouse embryonic fibroblasts (mEFs). Understanding the composition of the conditioned media is important as it is used to support the growth of human embryonic stem cells *in vitro*.<sup>2</sup>

### Methods

**Cell Culture** - supernatant conditioned by mEFs for 24hrs, stimulated with 8ng/ml of basic fibroblast growth factor (FGF-2) was analyzed. Initial media composition was DMEM supplemented with 20% knockout serum replacement (KOSR) media (Gibco/Invitrogen). **Sample preparation** - concentration of protein in the media was assessed by a standard Bradford assay. Before IEF or C4 separation proteins were denatured, reduced, and alkylated. Conditioned media samples were diluted 1:3, and made up to 6M urea, 50mM ammonium bicarbonate. They were then reduced with 20mM DTT for 45min at 37°C and reduced with 40mM iodoacetamide for 45 min at 37°C. **Iso-electric Focussing (IEF)** - in-solution, iso-electric focusing on a ZOOM IEF fractionator (Invitrogen) using all 5 fractions from pH 3-10, according to standard protocol.<sup>3</sup> Samples were focused until the current reached 0.2 mA @ 600V and all the bromophenol blue tracking dye had migrated into the anode chamber. 2mg of total protein was separated at a time. The corresponding pH fractions from 3 separate isolations were pooled for a total of 6mg of protein. **Albumin Affinity depletion** - Montage albumin depletion kit (Millipore) standard protocol. **C4 reverse phase HPLC** - (Agilent 1100 HPLC system) using a Synchropak C4 - RP4 gold column with 250 X 4.6mm ID. 0.5mg of total protein was injected per run, and separation was repeated 12 times. Corresponding fractions from consecutive injections were pooled for a total of 6mg of protein. Samples were eluted using a linear 22 min gradient from 5-95% acetonitrile with 0.1% trifluoroacetic acid. Albumin consistently eluted between 12 and 15 min. **LC-MS/MS analysis** - samples were digested with porcine modified trypsin (Promega), and analyzed by nano flow LC-MS/MS (Q-ToF Global, Micromass/Waters). Peptides were separated on a 15cm X 75µm C18 column (LC Packings) using a 90min gradient. Each sample was injected twice, with the second injection utilizing an exclusion list based on peptides identified in the first. **Bioinformatics** - each of the data sets was analyzed by Mascot (Matrix Science), Peaks version 2.2 (Bioinformatics Solutions), and Protein Lynx Global server (PLGS) 2.0 (Micromass / Waters). The tolerance on the search was set to 50ppm for the peptide mass and 0.1Da for the fragment ions. Positive IDs were found in Mascot entries with scores greater than 30 and Peaks or PLGS entries with confidence scores of 80% or greater. All included entries with confidence values of less than 95% have had their MS/MS spectra validated manually.

<b>Table 1:</b> Approximate and relative concentrations of the protein component of the Knockout Serum Replacement reagent.			
Protein Component	Approximate Concentration*	Concentration used at 20%	Concentration relative to BSA
Bovine Serum Albumin	83 mg/ml	17 mg/ml	1/1
Transferrin	0.055 mg/ml	11 ug/ml	1/1500
Insulin	0.1 mg/ml	20 ug/ml	1/850
*Stock concentrations have been interpreted from the existing patent on the serum replacement reagent <sup>1</sup>			

## Results

- 1D gel combined with MS analysis, and published formulation show the major component of the KOSR reagent to be BSA, estimated to account for greater than 99% of the total protein content.
- The majority of the BSA can be isolated to a single fraction by either C4 reverse phase chromatography or in-solution isoelectric focusing. Affinity removal of BSA failed to remove sufficient amount of BSA to make a difference in MS analysis.
- By LC-MS/MS analysis, more than 20X more peptides (not including those due to BSA) were identified by either C4 or IEF separation when compared to no removal of albumin.
- There was a total of 45 proteins identified:
  - 32 were bovine / sheep serum proteins – presumably artifacts of the BSA and transferrin used in the KOSR media which are both affinity purified from whole serum.
  - 13 were putative *Mus musculus* proteins that could be secreted by the mEFs in culture.
- The use of this method allows for a greater than 1500 fold dynamic range in concentration of proteins identified (relative to BSA) as shown by the identification of transferrin in both the C4 and IEF
- The use of both IEF and C4 separation are also complementary to each other, shown by proteins uniquely identified in each method. Many of the proteins identified by the C4 separation had isoelectric points outside that of the fractions analyzed by IEF. One specific example is that if Insulin A chain which was identified in the 2<sup>nd</sup> C4 fraction, but not found in the IEF fractions as its PI (2.2) was outside the effective range of separation
- One interesting mouse protein identified was a 10 kDa predicted secreted protein (AAQ88556). Further investigations will be done to see the effect of this protein in influencing embryonic stem cell growth.

## References:

1. Price PJ, Goldsborough MD, & Tilkin ML. (1998) ES cell serum replacement. International Patent Application WO98/30679.
2. Xu C *et al.* (2001) Feeder-free growth of undifferentiated human embryonic stem cells. *Nat Biotech.* 19: 971-74.
3. Zoom IEF instruction manual – Version A. (May 2003). Invitrogen, Carlsbad, California.

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