

INDUSTRIAL MUDPIT: AUTOMATED 4D NANOLC MS/MS OF PROTEOME SAMPLES

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INTRODUCTION

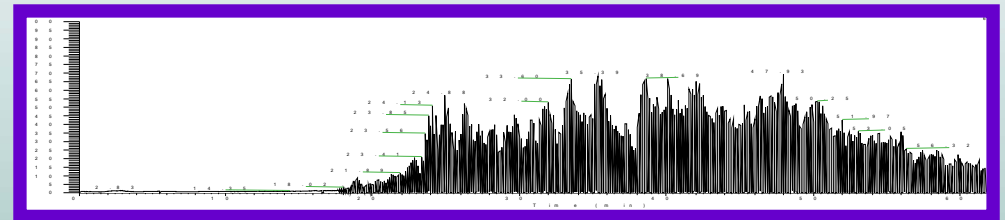
Although 2D peptide nanoLC/MS/MS (MUDPIT), which was introduced by John Yates at the 2000 ASMS meeting, is now a routine proteomics tool in many labs around the world, it does have several caveats that limit it from being more widely used. The biphasic SCX/RP nanocolumn used in conventional MUDPIT has limited sample capacity, allows salts into the MS ion source and requires the loading of concentrated, salt free samples.

Several researchers and instrument manufacturers have introduced methods and systems which overcome some of these limitations, but no system to date has solved all of these problems effectively. This study looks at a simple 4D LC system that overcomes all of these limitations and makes this technique routine for any proteomics laboratory.

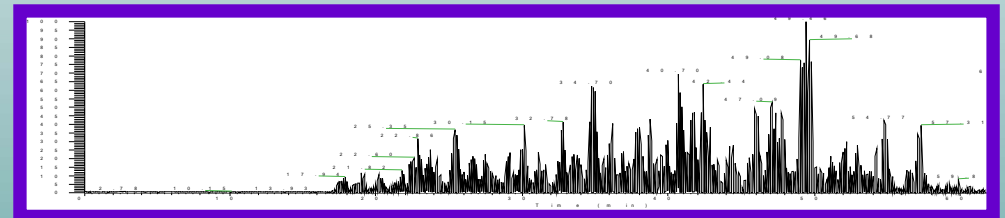
IMPACT OF SALT ON 2D MUDPIT METHOD

Yeast Proteome Peptides Not Retained on SCX Column

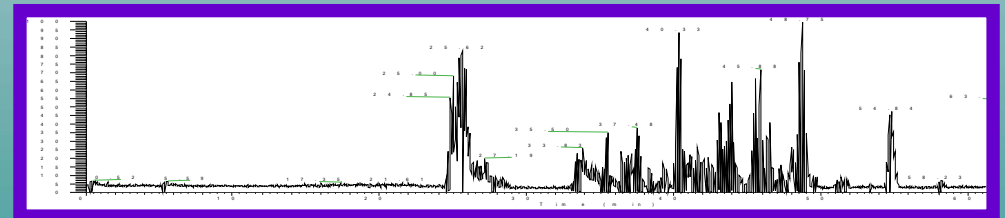
Digest Loaded in 1M Urea
50mM Ammonium Bicarb



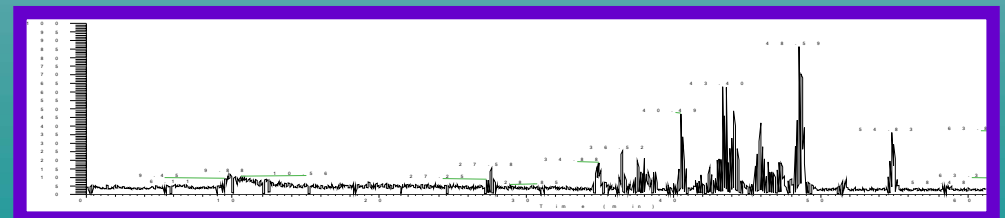
Digest Loaded in 50mM
Ammonium Bicarb



Digest Loaded in 10mM
Ammonium Bicarb



Digest Loaded in 0mM
Ammonium Bicarb



EXPERIMENTAL

Autosampler		Michrom Paradigm AS1BR Biocompatible Refrigerated AS
MDLC		Michrom Paradigm MS4B 100% Metal Ion Free Fluid Path
Columns	D1	3 x 10 mm Peptide Macrotrap
	D2	1 x 10 mm SCX Microtrap
	D3	150u x 50 mm Peptide Nanotrap
	D4	75u x 150 mm Magic C18
MS		Michrom Nanotrap Platform Thermo LCQ Deca ITMS

METHODOLOGY

Using an autosampler with sample preparation capabilities together with a multi-dimensional separations module with four pumps and two switching valves, we were able to test many variables in developing an easy to use and robust version of the Mudpit method.

This system allowed us to use four columns to (1) concentrate and desalt 50-500ul of protein digest on a RP peptide macro trap column, (2) trap and stepwise elute peptides from a SCX peptide micro trap column, (3) concentrate and desalt the SCX fractions on a RP peptide nano trap column and (4) gradient elute peptides on a 75u C18 nano column coupled to nanospray ESI/MS/MS.

Although complex in design, this setup and the software which controls the AS/MDLC/MS allows users to simply load salty digests and get good results routinely.

D1 (PMT) ONLINE DESALT DIGEST SAMPLE

The Paradigm AS1BR loads up to 500 ul of digest (Yeast Proteome) on to a 50 ul Peptide Macro Trap (PMT) and the sample is desalted online with RP-A at 100-200 ul/min (1-5 min)

The Paradigm AS1BR then loads 100 ul of RP-B and the peptides are eluted from the PMT to the SCX micro trap column (2 min)



Paradigm AS1BR

D2 (SCX) SALT STEP ELUTION OF PEPTIDES

Most of the peptides from the 50ul D1 PMT retain on the 5ul D2 SCX micro trap and the RP-B is then flushed from the SCX micro trap with RP-A at 100-200 ul/min (1-2 min)

The AS1BR then loads 50 ul of each salt step (NH_4HCOOH) to elute peptide fractions from the SCX micro trap to the D3 Peptide Nano Trap (PNT)



Paradigm MS4B MDLC

D3 (PNT) DESALT PEPTIDE FRACTIONS

Each of the peptide fractions from then D2 SCX micro trap are retained on a 400 nl D3 Peptide Nano Trap (PNT) and the salt is then flushed from the PNT with RP-A at 50 $\mu\text{l}/\text{min}$ (2-5 min)



Paradigm Nanotrap Platform
For Thermo Nanospray MS

D4 (NRP) LC-MS/MS OF D2 SCX STEPS

Each desalted peptide fraction retained on the D3 PNT is then analyzed by LCMS/MS on the D4 Nano Reverse Phase (NRP) column (75 μ x 100mm 5 μ 200A Magic C18) in 30-120 min at 100-400 nl/min



Thermo LCQ Deca ITMS

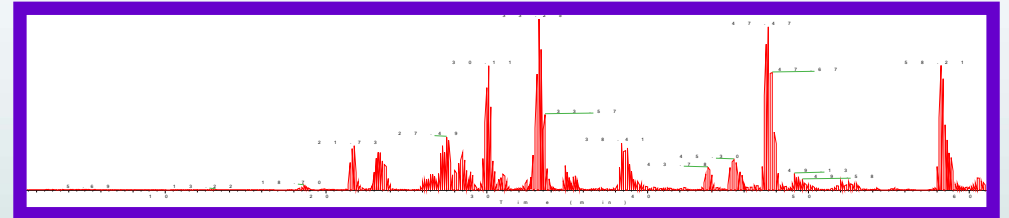
REPRODUCIBILITY OF 4D METHOD

500 Femtomoles
of BSA Digest in
500ul of 1M Urea

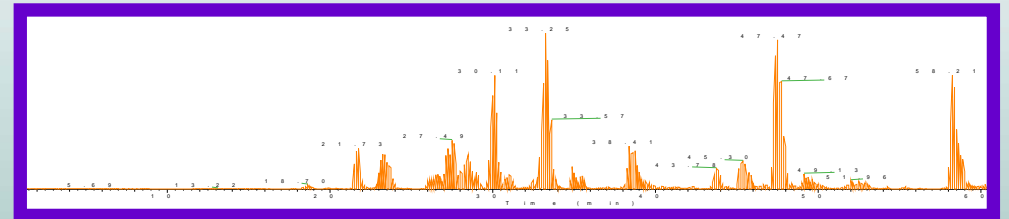
SCX Step 3 of 8
100 mM
NH₄HCOOH

60 Min Runs
8 Hours Apart
Over 40 Hours

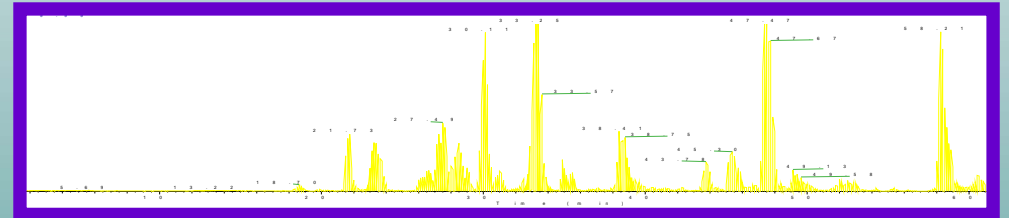
Run
1



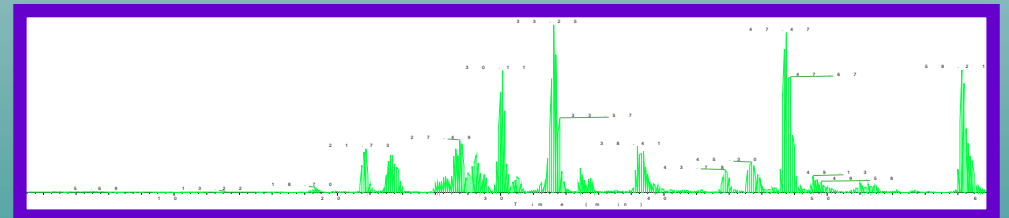
Run
2



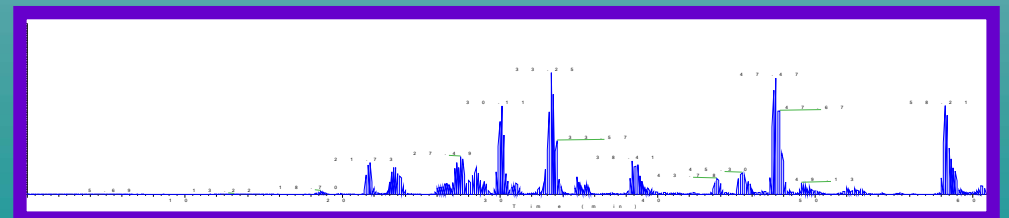
Run
3



Run
4



Run
5



DYNAMIC RANGE STANDARD (DRS)

Michrom has developed a Dynamic Range Standard (DRS) to use in methods development and validation of MDLC MS proteomics applications. This standard (P/N PTD/00001/52) consists of five yeast proteins mixed and digested as follows:

Protein	MW (daltons)	Amount (fmol/tube)
Alcohol Dehydrogenase	36,805	500,000
Enolase	46,784	50,000
Glutathione Reductase	51,195	5,000
Hexokinase	53,720	500
Phosphoglucose Isomerase	61,281	50

4D RESULTS FOR 5 YEAST PROTEIN DRS

The Michrom five yeast protein DRS was run by 4D LC/MS/MS using 5-20 SCX steps and RP nanoLC run times from 30-120 min, and the resulting MS/MS data was analyzed by Sequest. In general, detection of the lower abundance peptides improved with increased resolution (at a cost of total analysis time), but all five proteins were identified in a 15 hour run (12 salt steps with 90 min RP nanoLC runs).

<u>Protein Identified</u>	<u>Acession Number</u>	<u>#Peptide Matches</u>	<u>Percent Coverage</u>
Alcohol Dehydrogenase	1168350	29	82
Enolase	119336	24	63
Glutathione Reductase	6325166	15	28
Hexokinase	6321168	8	12
Phosphoglucose Isomerase	6319673	2	3

SUMMARY

The 4D LC MS/MS protocol described in this presentation is a very reliable and robust method for identifying proteins in complex proteome samples. This protocol allows the researcher to go directly from digestion to analysis without the need to concentrate or desalt samples.

The 4D Industrial MUDPIT system improves detection of low abundance proteins by allowing a very large sample loading (up to 500 ug of total protein digest in 500 ul) which can then be fractionated prior to nano LC/MS/MS analysis.

The 4D system can be used on a wide range of complex proteome samples including ID of trace proteins in 1D or 2D gels, differential protein expression studies to ID biomarkers and shotgun proteomics to characterize total system proteomes (as shown in the previous slide where > 2500 yeast proteins were identified in less than 24 hours).

CONCLUSIONS

- Industrial MUDPIT Provides Robust Proteomics Analysis
- 4D LC MS/MS Automates Analysis From Digestion to ID
- 4 Column System Maximizes Loading and Minimizes Time
- Online Automation Minimizes Sample Losses
- 4D LC MS/MS Improves Dynamic Range of Protein IDs
- Complete System Control in Xcalibur Insures Reliability
- Versatile Protocol Works on Broad Range of Samples