



# CAPRION PROTEOMICS

## *EVALUATION OF THE SUPERMIX DEPLETION COLUMN*



***REVEALING DIFFERENCES IN THE PROTEOME***

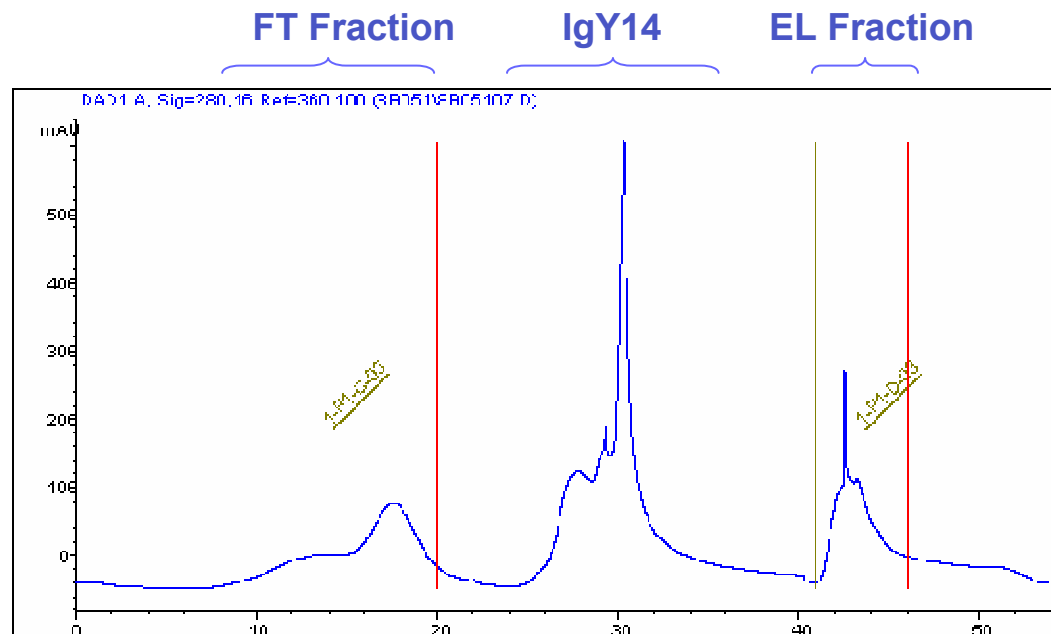
## PRESENTATION OUTLINE



- Overview of the newly developed SuperMix workflow
- Results from the Spiking experiment (Six Protein Depletion vs. SuperMix)
- Results from the Alzheimer's Disease biomarker study (Six Protein Depletion vs. SuperMix)

# SUPERMIX DEPLETION METHOD

- Depletion of plasma samples by IgY14 and SuperMix in a single run
- Total run time: 55 min
- Wash buffer: 150 mM Ambic pH 7.4
- Neutralization buffer (between Wash and Elution buffers): 100 mM Tris-HCl, pH 8
- Elution buffer: 100 mM Glycine-HCl pH 2.5
- Automatic collection of Flow Through (FT) and Eluate (EL) fractions
- Depletion of 75uL of plasma: ~ **40ug** of protein for FT and ~**200ug** for EL fraction



# MODIFICATION OF CELL CARTA WORKFLOW FOR SUPERMIX DEPLETION



- **Six Protein Depletion Workflow:**
  - 8 SCX fractions
  - 25 min LC/MS gradient; 500 um column (5000 peptides per run)
  - 8 ug per LC/MS injection
  - Total protein amount needed:  $8 \text{ SCX} * 8\text{ug} * 3 \text{ MS runs} = 192 \text{ ug}$
  
- **SuperMix Depletion Workflow:**
  - Analysis of FT and EL fractions
  - 80 min LC/MS gradient; 500 um column (15000 peptides per run)
  - 20 ug per LC/MS injection
  - Total protein amount needed:  $1 \text{ LC/MS} * 20\text{ug} * 3 \text{ MS runs} = 60 \text{ ug}$

## TIME SAVINGS OF SUPERMIX WORKFLOW (60 samples)

- # of sample processing steps:
  - Six protein Depletion: 9
  - SuperMix: 5
- Total sample processing time:
  - Six protein Depletion: 13 days
  - SuperMix: 6 days
- Total analysis time (Sample processing + LC/MS):
  - Six protein Depletion: 20 days
  - SuperMix: 11 days



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# COMPARISON OF SIX PROTEIN DEPLETION AND SUPERMIX WORKFLOW: SPIKING EXPERIMENT



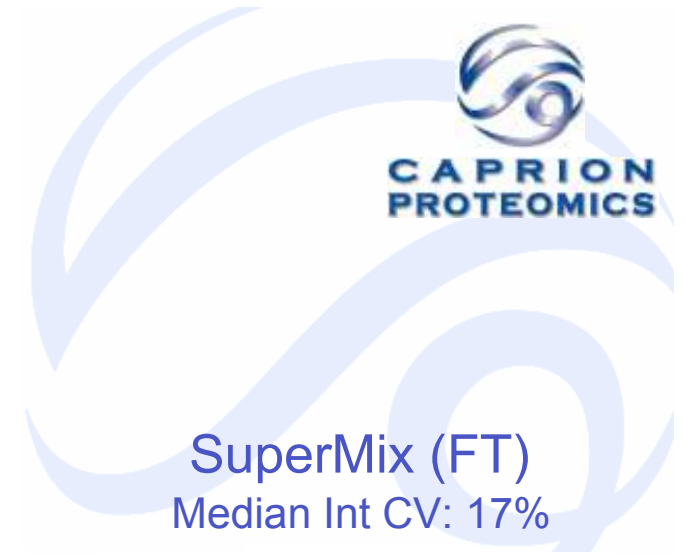
## Sample:

- 5 proteins spiked into a pooled human plasma sample
  - Ovotransferrin, Alcohol Dehydrogenase, Phosphorylase B, Beta-galactosidase and Hexokinase
- 8 different concentrations: 1 pg/uL to 100 ng/uL
- Processing and analysis of each sample in triplicate

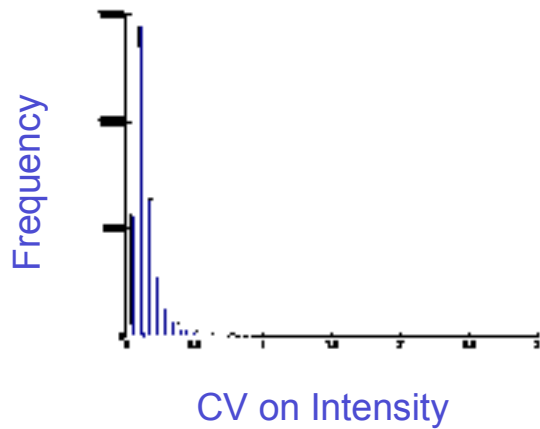
## Experimental:

- Six Protein Depletion → SCX (8 fractions) → LC/MS (25 min)
- IgY14 + SuperMix → LC/MS (80 min, FT Fraction)
- IgY14 → LC/MS (80 min)

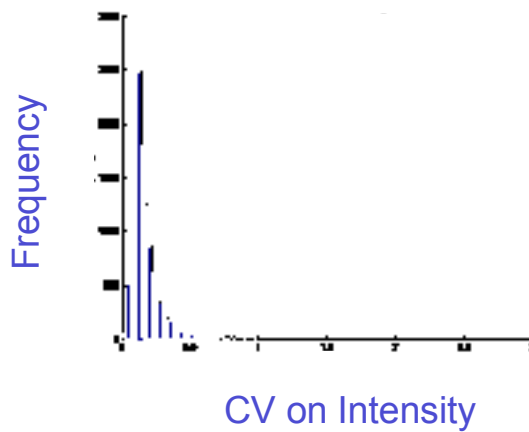
# INTENSITY CV OF DETECTED STUDY PEPTIDES (SPs)



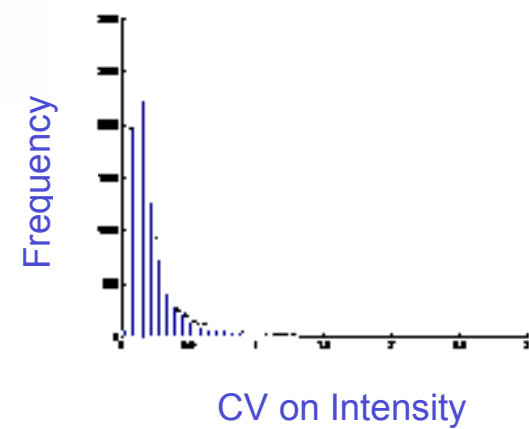
Six Protein Depletion  
Median Int CV: 12%



IgY14  
Median Int CV: 13%



SuperMix (FT)  
Median Int CV: 17%



- Low intensity CV variation for SPs from Six Protein Depletion and IgY14
- Slightly higher intensity CV variation for SPs from SuperMix FT fraction

## NUMBER OF SPIKED PEPTIDES IDENTIFIED

# of spiked peptides identified	6-Prot	IgY-14	SuperMix (FT)
100 ng/uL	159	65	216
25 ng/uL	120	51	179
5 ng/uL	65	40	133
1 ng/uL	4	6	24
200 pg/uL			4

- SuperMix identified the highest number of spiked peptides
- SuperMix allows the detection of ~5 times less abundant spiked proteins

## SUMMARY: SPIKING EXPERIMENT



- Intensity CV variation of detected peptides is slightly higher for the SuperMix workflow (Six Protein Depletion: 12%; SuperMix: 17%)
- Compared to Six Protein depletion samples with SCX fractionation, depletion with the SuperMix column and without SCX fractionation allowed the detection of more spiked peptides and ~ 5 times less abundant proteins.
- Eliminating SCX fractionation reduces the number of sample processing steps and sample processing time by about half.
- Additional fractionation (e.g. SCX) would decrease the SuperMix LOD further.

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# ALZHEIMER'S DISEASE BIOMARKER STUDY

## Goal:

- Comparison of Six Protein Depletion and SuperMix workflows in a Biomarker study

## Experimental Design:

- Analysis of 40 Alzheimer's Disease (AD) biomarker study samples with the SuperMix workflow
- Comparison of data to the Six Protein Depletion workflow data set acquired previously



10 healthy  
Volunteers (NEC)



10 patients with  
MCI



10 untreated  
AD patients

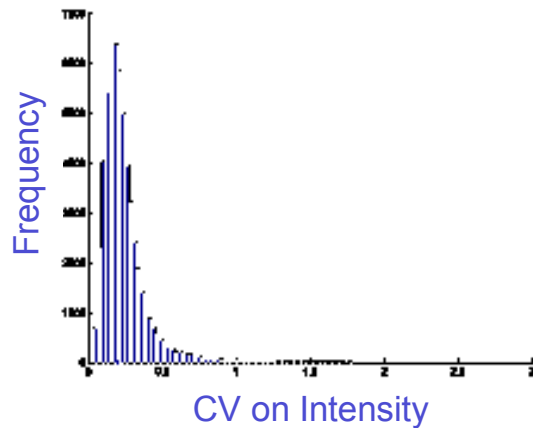


10 donepezil treated  
AD patients

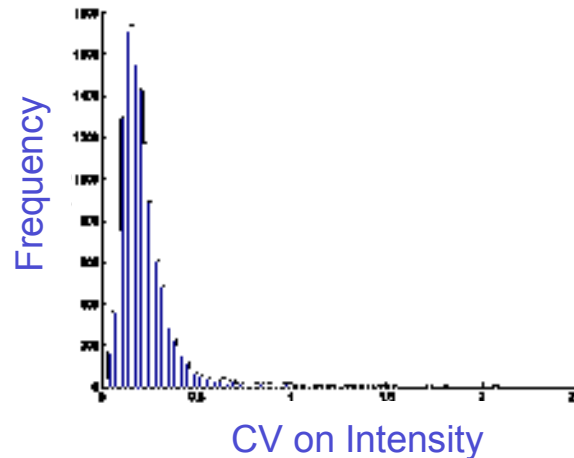
# INTENSITY CV OF DETECTED STUDY PEPTIDES (SPs)



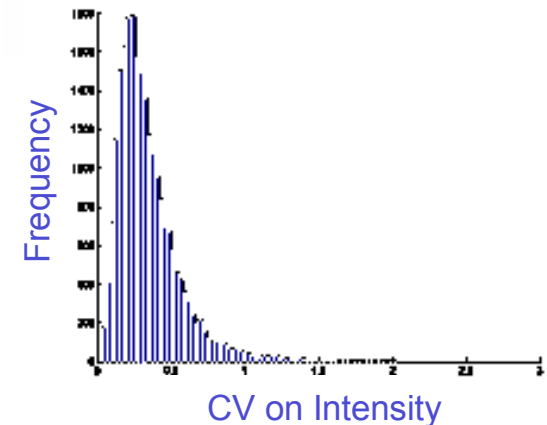
Six Protein Depletion  
Median Int CV: 21%



SuperMix (EL)  
Median Int CV: 19%



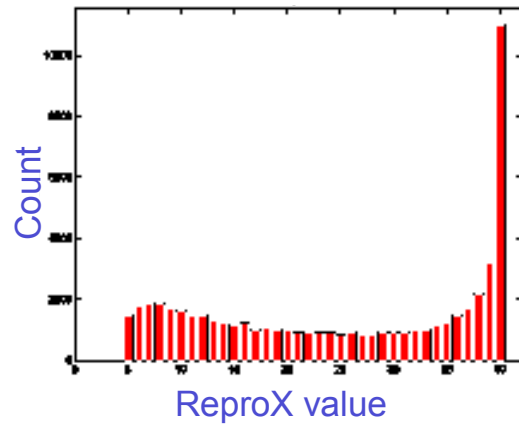
SuperMix (FT)  
Median Int CV: 30%



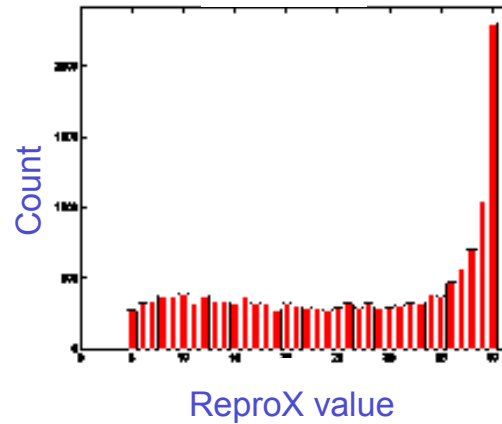
- Low intensity CV variation for SPs from Six Protein Depletion and SuperMix EL fraction
- Higher intensity CV variation for SPs from SuperMix FT fraction

# STUDY PEPTIDE REPRODUCIBILITY

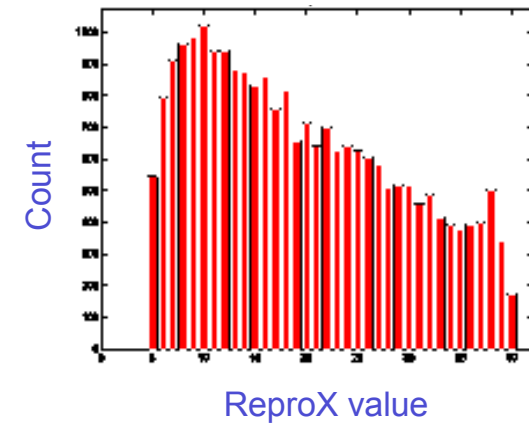
Six Protein Depletion



SuperMix (EL)

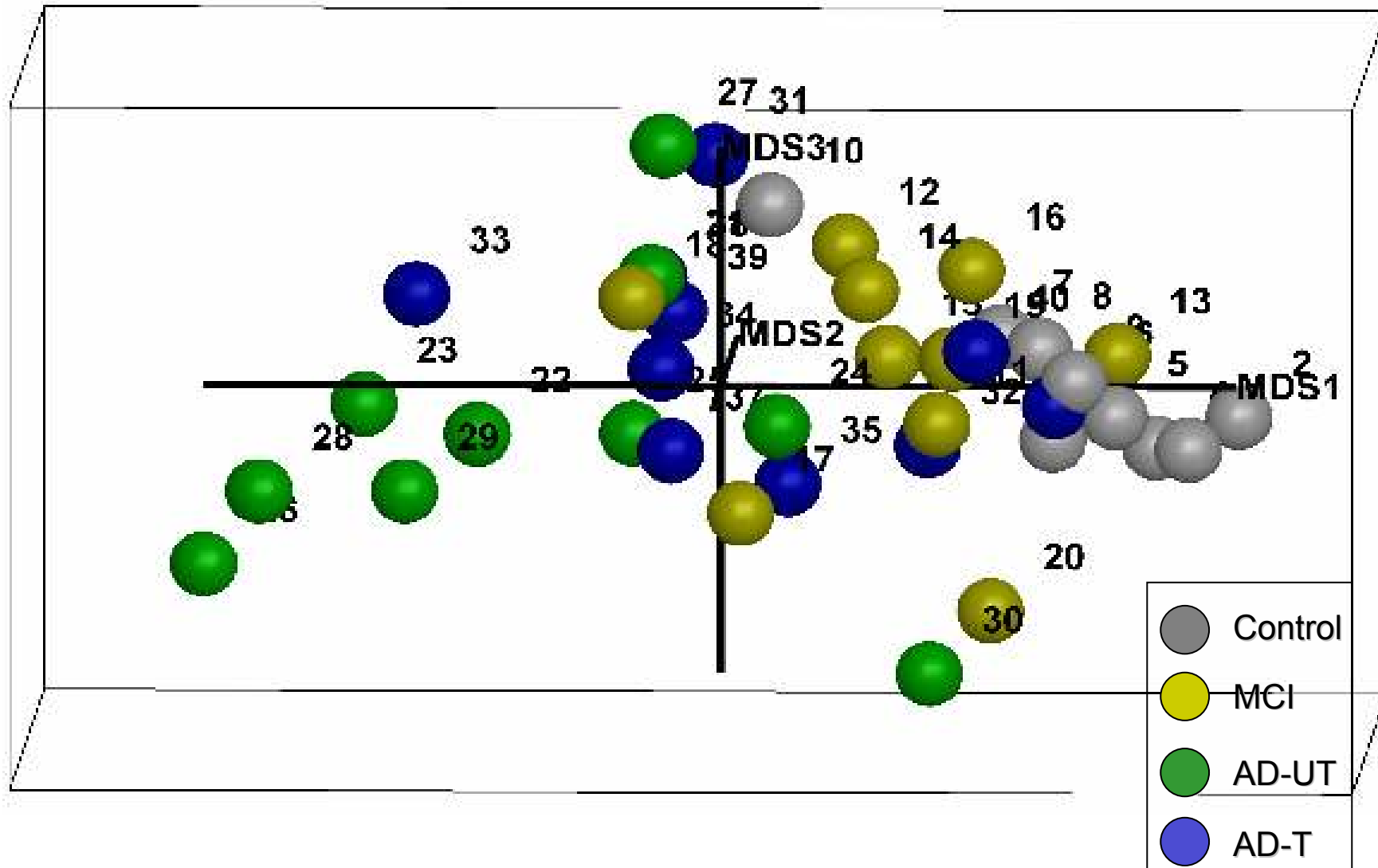


SuperMix (FT)

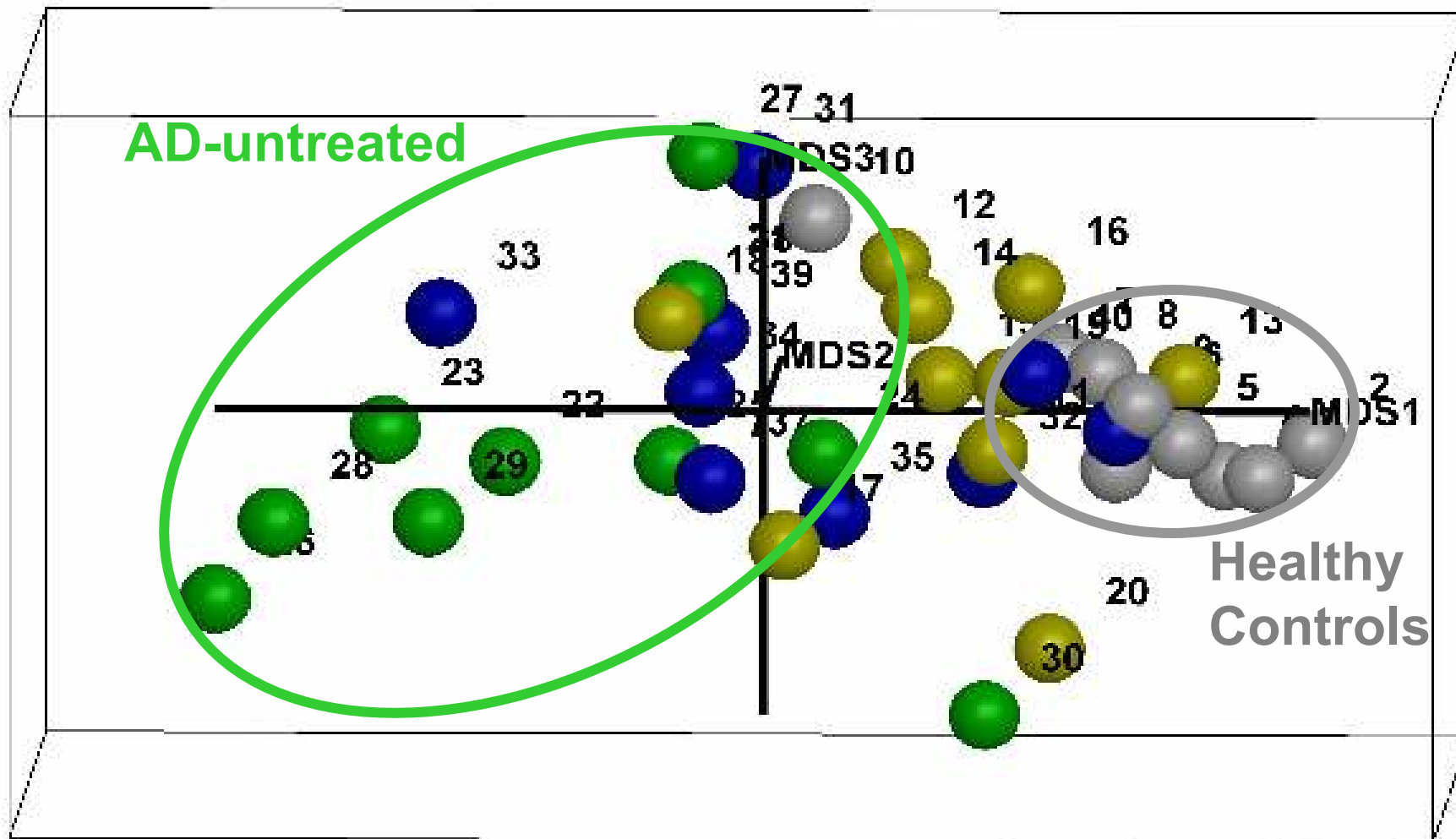


- Similar SP reproducibility for Six Protein Depletion and SuperMix EL
- Poor SP reproducibility for SuperMix FT fraction

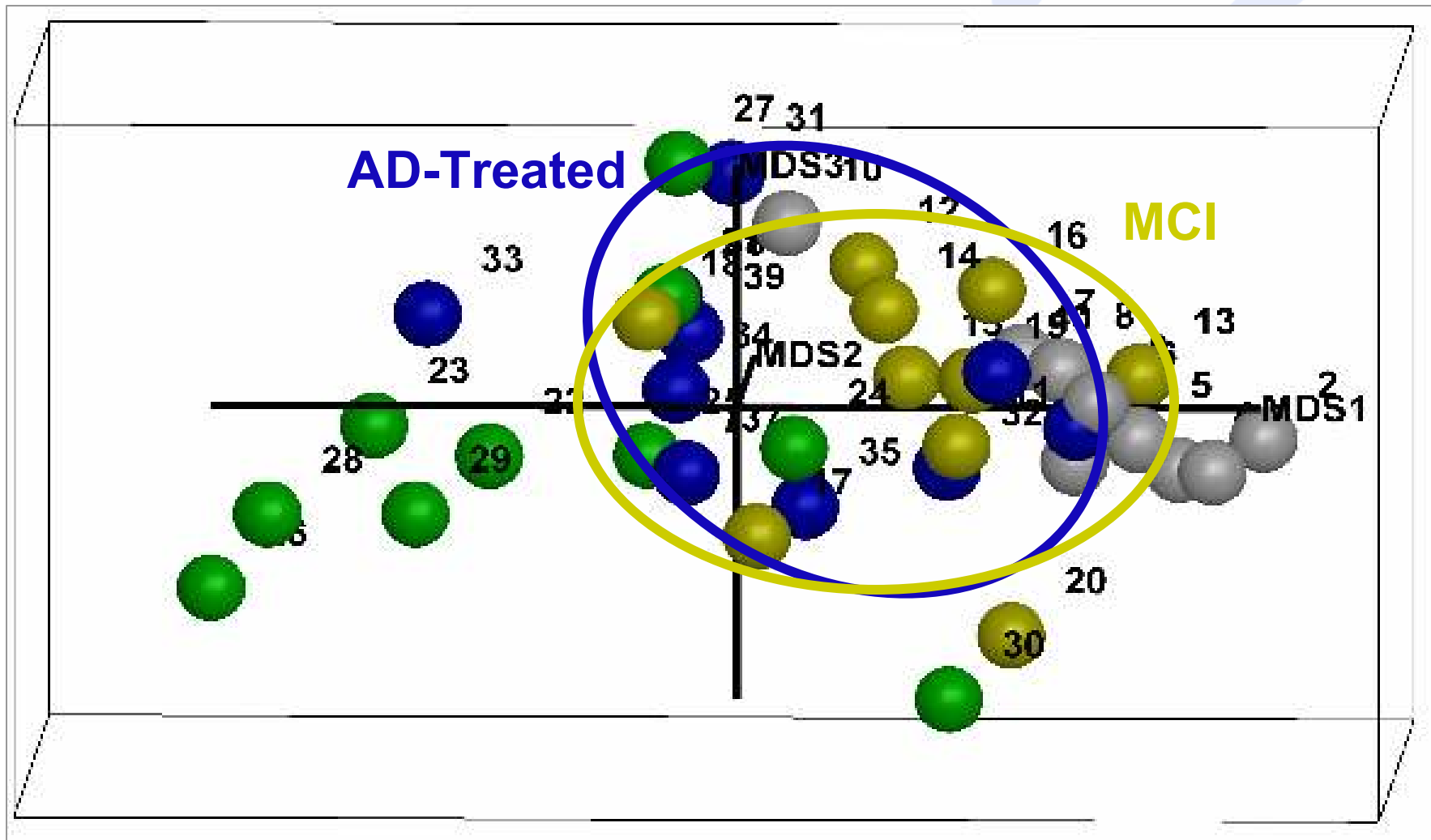
# MDS PLOT OF SUPERMIX (FT): DIFFERENTIALLY EXPRESSED PEPTIDES ONLY



# HEALTHY CONTROLS & AD (untreated) ARE WELL SEPARATED

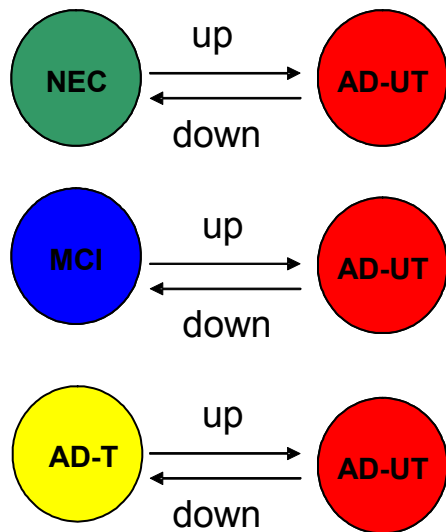


# TREATED PATIENTS AND MCI GROUPS FALL BETWEEN HEALTHY AND UNTREATED PATIENTS



# AD BIOMARKER STUDY: EXPRESSION ANALYSIS RESULTS

- $dl = 1.5$ ,  $p\text{-value} = 0.05$ , overexpressed  $\geq 7$  out of 10 times



	SuperMix		
	6-Prot	EL	FT
NEC	305	92	270
MCI	110	21	216
AD-T	42	36	34
AD-UT	142	134	223
	110	61	152
<b>Total:</b>	<b>814</b>	<b>370</b>	<b>1222</b>

# SUPERMIX WORKFLOW: IDENTIFIED PROTEINS WITH KNOWN ALZHEIMER'S DISEASE LINK



- **Acute Phase Proteins:**
  - Properdin
  - $\alpha$ 2-macroglobulin
    - Common variant leads to increased risk of AD
  - Complement factors
  - $\alpha$ 1-antichymotrypsin
    - Associated with pathogenesis of AD
    - Enhances formation of amyloid fibrils
  - Mannose binding protein
- **Calmodulin**
  - Associated with damage due to Ca ion dysregulation in AD
- **Carbonic anhydrase**
  - Previously identified as AD marker
- **Clusterin**
  - Co-localizes with fibrillar deposits in AD
- **Serotransferrin**
  - Previously identified as AD marker
- **Transthyretin**
  - Deposits in amyloid fibrils

# SUMMARY OF AD BIOMARKER STUDY

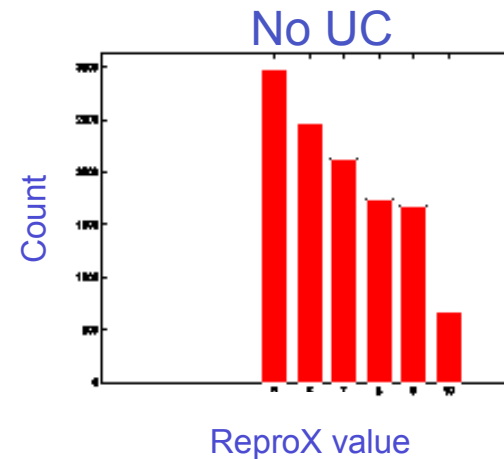
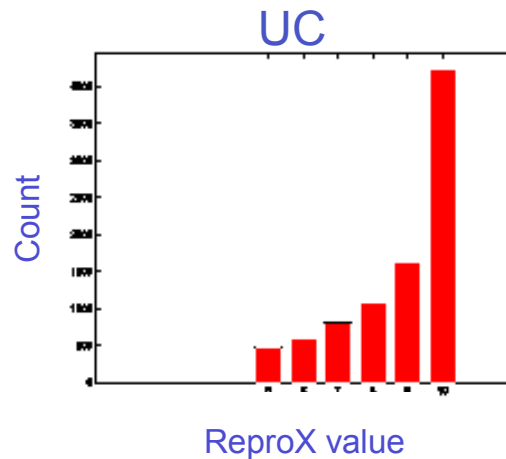


- Approximately 50% more differentially expressed peptides were detected with the unfractionated SuperMix depletion (FT fraction) than with the Six Protein depletion and fractionation method.
- A further increase in the number of differentially expressed peptides is possible by combining the eluate and flow through fractions from the SuperMix column.
- Proteins with known Alzheimer's Disease link were identified for the SuperMix workflow.
- Peptide irreproducibility of the SuperMix FT fraction might result in a higher false positive rate for differential expression.

# REMOVAL OF LOW MOLECULAR WEIGHT COMPOUNDS BY ULTRA-CENTRIFUGATION

- Reprocessing of 10 samples
- Ultra-centrifugation (UC) of samples with Ultrafree-MC Centrifugal Filter Units (5 kDa cut off; Millipore) prior to digestion
- **Results:**
  - Median peptide intensity CV decreases from 30% to 22% with UC
  - Improved peptide reproducibility

UC Filters



## CONCLUSION

- SuperMix workflow has several advantages compared to the Six Protein Depletion workflow:
  - Allows the detection of lower abundant plasma proteins
  - Reduces the number of sample processing steps and sample processing time by about half
  - Allows the identification of more proteins (excl. MSMS; Data not shown)
- Size of SuperMix depletion column should be increased to have sufficient protein amount for backup plates and MRM assays
- Removal of low molecular weight compounds prior to digestion seems to solve the peptide irreproducibility problem observed for the AD biomarker study.



**CAPRION  
PROTEOMICS**