**Biotin Hydrazide Capture of Carbonyl Adducted Proteins**

**Ethanol:Methanol:Chloroform Precipitation**

**In Gel Peptide Digestion & Extraction**

**MS Sample Prep & Analysis**

**Day 1**

* Prepare Lysis Buffer
	+ Prepare a fresh biotin hydrazide (BH) stock at 50mM in DMSO
		- Biotin hydrazide powder stored dessicated at 4oC
		- Sample calculation for 1mL at 50mM:

258.34g/mol \* 1mol/1000mM \* 50mM = 12.917g/L

12.917g/L \* 1L / 1000mL \* 1mL = 0.0129g or 12.9mg

Add 1000uL of DMSO to 0.013g of BH and heat at 80oC for about 2 minutes to get completely into solution…

* + Add 50mM BH stock to TNEB for a final concentration of 5mM
		- 4mL of TNEB
			* 400uL of 50mM BH stock
			* 4uL of Protease Inhibitor (PI) to a final of 1:1000
			* 3.6mL of TNEB
* Remove brain, place into a 15mL conical tube and immediately add 1mL

of ice cold TNEB (+BH and PI)

* + Start timer as soon as BH is added (this time will count towards the

2hr BH incubation)

* Transfer to a glass dounce and homogenize for ~30 strokes on ice
* After douncing, sonicate at 6 watts for 10 seconds
* Pass the lysate through a 40 micron cell strainer
* Immediately determine protein concentration of each sample
	+ Note: You cannot use the BCA kit to do this as the BH interferes

with the absorbance readings.

* + - Instead, use the NanoDrop to measure the [protein]
* Prepare each sample at a final of 4mg total protein in 2mL of TNEB (+BH

and PI) – i.e. no more than 2mg in each mL

* + Cap and invert tubes to mix well
	+ Wrap caps with parafilm
	+ Place tubes on Labquake in cold room and rotate for the remaining time of the 2hr total BH incubation
* While incubating, locate sodium borohydride (NaBH4) stock solution (2M) or prepare more if needed...
* Immediately following the 2hr incubation in BH, add 25uL of 2M NaBH4 to each sample tube
	+ Cap samples
	+ Vortex quickly
	+ Incubate for 30’ at room temperature with lids open as the reduction reaction produces gas in the form of bubbles
		- Final NaBH4 concentration = 50mM
* During the 30’ reduction reaction, place ethanol (EtOH) and methanol (MeOH) on ice.
* After the reduction is complete, Add 3mL of ice cold EtOH
* Vortex
* Spin at 3000rpm for 10 minutes
* Remove supernatant and add 1mL of ice cold MeOH and 2mL of ChCl3 to wash/re-suspend the pellet
	+ Vortex well
	+ Spin down at 2000rpm for 10 minutes
* Remove supernatant and wash/re-suspend pellet in 1mL of MeOH
	+ Transfer to a microcentrifuge tube
	+ Vortex
	+ Spin at 5000rpm for 5 minutes
* Remove supernatant and wash/re-suspend pellet in 1mL of MeOH
	+ Vortex
	+ Spin at 5000rpm for 5 minutes
* Remove all supernatant and re-suspend in 1mL of 0.5% SDS
	+ Sonicate sample at 6 watts for 10 pulses
	+ Heat for 10 minutes at 95 oC (Solution will become clear)
* Prepare an input sample
	+ 8uL of 4X Sample Loading dye
	+ 2uL of 1M DTT
	+ 15uL of the lysate
		- This equals 1% of the sample and will be run on the WB
		- Vortex well
		- Boil for 10 minutes at 95oC
		- Store at -20oC
	+ Take an aliquot for protein assay
		- To see what is left versus what you started with (4mg)
			* Again, use the NanoDrop
* Prepare streptavidin beads for pull down
	+ For each sample, add 400uL of bead slurry to a microcentrifuge

Tube (This is ~200uL of actual beads)

* + - Note the lot #:
	+ Spin beads slurry down in benchtop microfuge
	+ Dump off supernatant = Ethanol the beads are initially stored in
	+ Add 1mL of PBS
	+ Spin, dump, add 1mL PBS – repeat for 3 washes

1 2 3

* + After final wash, remove as much of the supernatant as possible
* Prepare sample tubes for overnight pulldown
	+ 8mL of 1X PBS
	+ 1mL of TNEB (+PI)
	+ Washed beads (re-suspend beads in 500uL PBS, add to conical

tube, wash micorcentrifuge tube with an additional 500uL and add to conical as well)

* + To these tubes, add the remaining brain lysate (minus the input

sample volume and a few uL for protein chack…)

* + - Parafilm the tubes and place on the Labquake overnight at

4oC in cold room.

**Day 2**

* Prepare Amicon filter top tubes by adding 3mL of 1X PBS to filter and spinning at 5000rpm for 15 minutes.
* Prepare elution buffer (30mM Biotin / 2% SDS in 1X PBS) as follows:
	+ - Sample calculation for 10mL
			* 244.31g/mol \* 1mol/1000mM \* 30mM = 7.33g/L
			* 7.33g/L \* 1L / 1000mL \* 10mL = 0.0733g or 73.3mg
				+ Add 7mL of 1X PBS to conical tube
				+ Add the 73.3mg of biotin to the PBS and vortex

pH to 12 in order to get biotin in

solution…

* + - * + Add 2mL of 10% SDS
				+ Bring up to 10mL total with 1X PBS
* Prepare fresh 4M Urea
	+ Calculations for 6mL:
		- MW = 60.06g/mol
			* 60.06g/mol \* 4M = 240.24g
			* 240.24g/1L \* 1L/1000mL \* 6mL = 1.44g
* Remove samples from cold room.
* Spin down at 5000rpm for 5 minutes to settle the beads.
* After spinning, remove supernatant and place into new appropriately labeled 15mL conical tubes.
	+ You will concentrate this sample (Flow through) in the Amicon

tubes by adding 3mL at a time to the filter top and spinning at 5000rpm for 15 minutes.

* + The goal is to concentrate down to less than 1mL
		- In the end, you will bring all sample tubes up to 1mL by

adding PBS – this way, your input and flow through samples are both taken from an intial sample of 1mL total volume.

* + Prepare Flow Through sample tubes as follows:
		- * 8uL of 4x loading buffer dye
			* 2uL of 1M DTT
			* 15uL of the concentrated and volume adjusted

sample…

* + - * + Vortex well
				+ Boil for 10 minutes at 95oC
				+ Place on ice
* Begin washing the beads by re-suspending them in any remaining buffer and transferring to a microcentrifuge tube.
	+ - Wash the initial tube with an additional 500uL of 1X PBS and

transfer this to the microcentrifuge tube as well (Goal = leave no bead behind).

* + - Spin down in benchtop microfuge for about 30 seconds.
* Remove supernatant via pipette (so as not to loose any beads).
* Add 1mL of 1% SDS to beads
* Vortex quickly
* Invert tubes ~20 times to wash well
* Spin down in microfuge for 30 seconds
* Repeat wash again with 1mL of 1% SDS

1 2

* Following the second 1% SDS wash, remove supernatant and add 1mL of

4M Urea to the beads.

* Vortex quickly
* Invert tubes ~20 times to wash well
* Spin down in microfuge for 30 seconds
* Following the 4M Urea wash, remove supernatant and add 1mL of

1M NaCl to beads.

* Vortex quickly
* Invert tubes ~20 times to wash well
* Spin down in microfuge for 30 seconds
* Following the 1M NaCl wash, remove supernatant and add 1mL of

1X PBS to beads.

* Vortex quickly
* Invert tubes ~20 times to wash well
* Spin down in microfuge for 30 seconds
* Repeat wash again with 1mL of 1X PBS

1 2

* Following the second 1X PBS wash, remove as much supernatant as

possible via pipette.

* Spin beads down again without adding any more solution and remove any

remaining supernatant via pipette

* Add 400uL (this should be the same volume as the bead slurry used) of

the elution buffer (30mM Biotin / 2% SDS in 1X PBS) to the beads and allow to incubate at room temperature for 30 minutes on rotator.

* Following incubation, boil sample at 95oC for 15 minutes
* After boiling, spin down the tubes in the benchtop microfuge for about 1

minute.

* To keep the sample clean of beads (and in turn streptavidin) remove the

supernatant and place into a new microcentrifuge tube fitted with a SpinX 0.45 micron Nylon filter top

* + Spin down at 5000rpm for 5 minutes
* Throw out the top of the filter unit containing the beads that were

accidentally collected.

* Add 1.2mL of Acetone to the sample and place at -80oC overnight to allow

the samples to precipitate.

* Prepare Eluate tubes as follows:
	+ 4uL of 4X loading buffer dye
	+ 1uL of 1M DTT
	+ 15uL of the concentrated and volume adjusted sample
		- Vortex well
		- Boil for 10 minutes at 95oC
		- Place on ice
* Once you’ve prepared your Eluate sample for the streptavidin WB check

(see below), place the remaining sample in the speed vac centrifuge for 15 minutes. This will decrease the sample volume so that the entire remaining eluate can be loaded into one lane on the gel for cutting.

* + If your remaining Eluate sample will fit in one lane already (i.e. is

less than 40uL) you can skip this step…

* + Prepare the Eluate sample to be run on the gel for cutting
		- To the remaining eluate sample, add:
			* 10uL of Loading dye
			* 2 uL 1M DTT
				+ Vortex
				+ Boil for 10 minutes at 95oC
* Run a WB gel containing the Input, Flow Through and Eluate samples from each condition.
* Transfer the gel unto PVDF and probe for Streptavidin
	+ The idea here is that you can see total adducted proteins your initial sample in the Input lane, the amount of adducted proteins that were not captured by the bead pull down (i.e. hopefully none) in your Flow Through lane and the final amount of adducted, captured proteins (that will go on to be analyzed by Mass spec) in your Eluate lane.

- Run a WB gel of the remaining eluate sample for each condition

* + This will be the gel that is cut and further processed for MS Analysis

- After running the gel, place into wash box, cover in water and incubate on

 shaker for 15 minutes

* Remove water wash and cover gel in simply blue safe stain
	+ Heat in microwave for about 30 seconds
		- Remove and shake by hand to redistribute the stain
	+ Place back in microwave for another 30 seconds
	+ After second cook, dump off safe stain and add water to de-stain

the gel

* + - Repeat water washes (to de-stain) once every 30 minutes for

a total of 4 washes.

* + - Following the 4th wash, add fresh water and place on shaker

overnight to completely de-stain.

**Day 3**

* Take a picture of the de-stained gel on the scanner inside a sheet protector.
* Place gel on the light box (again on a sheet protector)
	+ Use metal spatula to cut off the molecular weight ladder and any

other excess gel (i.e. empty lanes, well walls, etc..)

* + In addition, cut out the large 19kDa band as this is Streptavidin and

will hinder the MS Analysis…

* Once all excess gel is removed, begin cutting horizontal fractions from the

lowest molecular weight band (Fraction 1) towards the highest (Fraction X)

* + Use a new razor blade for each fraction and for each genotype
	+ If you can cut each blue band of protein individually, do so, if not, it

is OK to combine two or three into one fraction – just try to keep them as slim as possible)…

* After you cut one fraction horizontally, use a razor blade to cut it one or

two more times horizontally and then vertically into as many 1mm pieces

as possible

* + Use the razor blade to scoop up the pieces and place into a

microcentrifuge tube labeled for the appropriate fraction

* + - Add 100uL of AmBic to each fraction tube as you go
			* Note: if your gel pieces are not entirely covered in

AmBic, add more as needed…

* + - Mix well to be sure all gel pieces are covered
		- Allow to sit in AmBic until all fractions are collected
* Once all fractions are cut, use an aspirator fit with a 21 Gauge needle to

remove AmBic from the gel pieces

* Replace with 100uL of fresh AmBic (or volume needed to cover all pieces)
* Add 5uL of 1M DTT (50mM final) to each fraction tube
	+ Note: If you used more than 100uL of AmBic to cover your samples

completely, you’ll have to adjust accordingly)

* + Vortex well
	+ Heat at 55oC for 20 minutes
		- The heat and DTT are used to break any disulfide bonds

and/or salt bridges that formed, reducing these bonds frees up the cysteine residues of the captured proteins

* While the fractions are incubating in DTT, prepare fresh iodoacetamide

(IAM) – powder stored in light protective box at 4oC

* + Note: IAM is extremely light sensitive – so prepare in dark and keep

out of light as much as physically possible. IMA is used to alkylate the cysteines that were freed up by the DTT/heating, essentially blocking them from forming or re-forming any disulfide bonds or salt bridges. This is important because it makes the trypsin digestion possible…

* + Sample calculation:
		- 184.96g/mol \* 1mol/1000mM \* 600mM = 110.97g/L
		- 110.97g/L \* 1L/1000mL \* 2mL = 0.2219g or 221.9mg
			* Add 221.9mg to 2mL of water
* Once samples are done heating, add 17.5uL of 600mM IMA for a final

[IAM] of 100uM and incubate in the dark at room temperature for 15 minutes.

* + Note: If you used more than 100uL of AmBic to cover your samples

completely, you’ll have to adjust accordingly)

* Following the IAM incubation, remove the supernatant with an aspirator fit

with a 21 gauge needle

* Add 100uL of a 1:1 mixture of 100% Acetonitrile:AmBic
	+ You may have to adjust this volume to cover gel pieces completely
	+ Vortex samples well and incubate at room temperature for 15

minutes

* Following incubation, remove the supernatant with an aspirator fit with a

21 gauge needle

* Add 100uL of a 1:1 mixture of 100% Acetonitrile:AmBic
	+ You may have to adjust this volume to cover gel pieces completely
	+ Vortex samples well and incubate once more at room temperature

for 15 minutes

* + Note: This wash is used to remove any remaining protein dye from

the gel fractions. Usually two washes are enough to get a clear (not blue) supernatant, if not, add a third wash

* Following the final Acetonitrile:AmBic wash, remove the supernatant with

an aspirator fit with a 21 gauge needle

* Add 100uL of 100% Acetonitrile
	+ You may have to adjust this volume to cover gel pieces completely
	+ Vortex samples really well
	+ Incubate samples at room temperature for 15 minutes
		- Note: The 100% Acetonitrile is used to dehydrate the

samples. When this process is complete (after the 15 minute incubation), you should be left with tiny, pure white cubes (Looks like the purest, baby sugar cubes you’ll ever see).

* Following dehydration, remove the supernatant with an aspirator fit with a

21 gauge needle

* + At this point you can store the crystallized gel pieces at -20oC or

continue to the trypsin digestion depending on whether or not the Mass Spec is free…

**Day 4**

- Trypsin Digestion

* Prepare 1ug/uL trypsin (100ug of Promega Trypsin Gold MS

Grade, Catalog #V528A) by adding 100uL of 50mM Acetic Acid

* Prepare 2mL (or more, as needed) of 0.01ug/uL trypsin as follows:
	+ 2mL AmBic
	+ 20uL of 1ug/uL trypsin stock
		- Aliquot remaining 1ug/uL trypsin (10uL each) and store at –

20oC

* Remove fractions from -20oC
* Add 50uL of 0.01ug/uL trypsin to each fraction tube
	+ 500ng [final trypsin]
* Then add 200uL of AmBic to completely immerse gel pieces
* Place fractions in 37oC shaker at 200rpm overnight to digest

**Day 5**

- Peptide Extraction

- Prepare extraction buffer as follows:

 - 60% Acetonitrile

 - 40% H2O

 - 1% Formic Acid (in fume hood)

 - So for 50mL

 - 30mL of acetonitrile

 - 20mL of H2O

 - 500uL of formic acid

- Add 200uL of extraction buffer to each fraction tube (already containing gel

 pieces and ~200uL AmBic/Trypsin

 - Vortex well

 - Incubate at room temperature for 20 minutes

- While incubating prepare a set of tubes to match the fraction sample tubes

- After 20 minute incubation is complete, use a P200 pipette to remove the

 supernatant (~350uL) and place into the new microcentrifuge tubes

- To the initial fraction tubes, add another 200uL of extraction buffer

 - Vortex well

 - Incubate at room temperature for 20 minutes

- Following second incubation, use P200 to remove the supernatant and add to

 the new microcentrifuge tube (Volume now = 550uL)

- To the initial fraction tubes, add another 200uL of extraction buffer

 - Vortex well

 - Incubate at room temperature for 20 minutes

- Following the third incubation, use P200 to remove the supernatant and add to

 the new microcentrifuge tube (Volume now = 750uL)

* + Note: At this point, the gel will begin to turn white again because all

of the peptides are being extracted and it is dehydrating…

- Throw away any remaining gel pieces and initial tubes

- Place all supernatant tubes into the Thermo VaporTrap centrifuge with the lids

 open and leaving a space between tubes so open lids to not cover neighboring

 tubes

* + Vent the VaporTrap for about 3 seconds by turning the blue dial to

the arrow

- Spin samples on “manual” setting for 3 to 4 hours

 - After 3 hours, check on samples:

- If not fully evaporated, allow to spin for another hour, check,

repeat, etc… until they are completely done…

- If fully evaporated (i.e. absolutely NO liquid remaining and just a

film-like substance in the bottom) remove the tubes from the centrifuge, cap the tubes and store at -20oC until LCMS if open for your run…

**Day 6**

* LCMS Analysis
* Prepare CPTAC2 buffer as follows:
	+ 2% Acetonitrile
	+ 0.1% formic acid
* Re-suspend peptides by adding 20uL of CPTAC2 buffer to each tube
	+ Vortex
	+ Allow to incubate at room temperature for at least 10 minutes to

undergo complete re-suspension

* While samples are re-suspending, label and assemble glass MS vials with

inserts

* Once re-suspended, transfer all 20uL of peptides to MS vials
	+ Be sure there are NO BUBBLES!
	+ Note: The MS injector will sample only 3uL, which means the

analysis is based on 15% of the total peptide sample

* Place samples into LC-MS que
	+ Label appropriately in the spreadsheet
	+ Add blank, BSA 10X standard, blank, BSA 1X standard, blank and

in the beginning of the shoot, in between different genotypes and at the end of samples

* + Group samples into batches
		- Blanks and Standards = batch 1
		- WT Samples = batch 2
		- Blanks and Standards = batch 3
		- KO Samples = batch 4
		- Blanks and Standards = batch 5
			* The LC-MS runs about 15 samples per day, so if you

are running over the weekend, add the Blanks and Standards batch the appropriate amount of time so the machine keeps cycling until you get in the following week…

* Record sample position in notebook
* Hit run

**Day 7**

* Sample Analysis
* Run output through ScanSifter to filter out any junk files (i.e. peaks without

a minimum of 10 counts per peak

* + This sorting typically takes about an hour to process
	+ Once complete, save the raw files from the instrument to the T drive
* Import the raw ScanSifter data into MyriMatch (Cluster Analysis)
	+ Choose the proper peptide database (mouse or human). This is an

entire library of millions of spectra from proteins in a database based on protein samples that were digested into tryptic peptides via *in silico* fragmentation

* + - i.e. all peptides that could exist are then matched to our

peptides

* + - Peptides are then assigned an ID based on this matching

process

* + - * Some IDs are confident IDs based on parsimonial

grouping

* + - * + These are grouped into related protein families
* Results from MyriMatch then go into ID Picker
	+ ID Picker takes all confident IDs and breaks them down into

Peptides, Peptide Groups, Proteins and Protein Groups

* + - Export this file (.tsv) into the same folder as the others
		- Open excel and then open the .tsv file
			* Export folder
				+ Spectra-per-protein-by-group.tsv
				+ SAVE as an excel file before you do anything

else!!!!!

* In excel:
	+ Take sequence ID column and sort by smallest to largest
		- Sequence ID of 1 = the most probable proteins of interest in

the samples

* + Move any sequences with an ID of 1 to a new sheet
		- Now change the name of the (/) column to total # of scans

and sort this column from highest to lowest

* + - * If you have a scan number = to or greater than the

number of conditions (i.e. 3 or greater if comparing WT, Het and KO) this means that there was at least 1 full scan across all three conditions for the same sequence… or in other words, all three conditions had the sequence representing the protein at least once

* Anything with a scan number less than your number of conditions should

be moved to a new sheet as these will be the most unique IDs

* + Now, sort by protein name alphabetically
		- If anything is labeled in the protein name column as CNTM

(contaminated) they will appear first in your list

* + - * Calculate the false positive potential as follows:
				+ Example:

444 total proteins

8 contaminants

1 reverse sequence

(CNTM + Rev) x 2 = X

Where 2 is an MS standard

(8+1) x 2 = 18

18 / 444 = 4%

You must be under 5% to

continue your analysis…

* Now, copy this sheet into a new sheet and delete all of the reverse hits

and contaminants = the total number of proteins ID (in this example, 435)

* + Now you can sort this data by the control column (or WT column)
	+ And then sort by the number of hits (largest to smallest)
		- Note: If you had 3 conditions, any scan number of 0 to 1

means the protein was definitely not detected in that sample… (i.e. a 1 may as well be a zero only 2 and up are considered actual scan hits)

* You can now take your protein ID column (sometimes labeled as IPI

column depending on the MS you use) and copy and paste into BioMart.org to get actual gene IDs

* + Go to BioMart.org
	+ Proceed to BioMart Portal
	+ Select ID Converter
	+ Paste the IPI list
	+ Choose the species data set needed
	+ Choose IPI IDs
	+ Choose Wiki gene names
		- Note: You can only do 50 to 100 proteins at a time and you

cannot have IPI: or the .#- in your pasted IPI list

* + Save file
	+ Open file in excel
		- Sort for significance
			* =IF(A2>2\*B2,1)
				+ Where A2 is the control column, > is greater

than 2\* is two fold, B2 is the treated columns and ,1 means excel will show a number 1 if this is true and the word false if it is not…