Maldonado Coral CompGO S. siderea

Subsetting S. siderea fasta for CompGO
We need to subset the full fasta file to include only proteins that were detected in the experiment.

Start with the Abacus output file.

1. Remove all proteins that are not from S. siderea. proteins start with “comp"
2. Remove proteins with ALL_NUMPEPSUNIQUE < 2. If only one unique peptide was detected in the experiment we do not have confidence that the protein is really “there”.
3. Save the list of protein IDs as a .txt file.
4. upload to the .txt file to your directory on the cluster. Log on to grid and go to a cluster node using qlogin.

From here, run a scrip that subsets your fasta file based on the list of proteins you just uploaded.

```
perl
/net/gs/vol4/shared/nunnlab/search/emmats/select_seqs/get_selected_sequences.pl -i
Sside.detected.proteins.txt -f
/net/nunn/vol1/emmats/databases/davies_Ssid_contam_symb.fasta -o
Sside.MSdetected.fasta
```

Parameters:
-i input file A Sequence ID List file that contains one sequence ID per line.
-f input file A FASTA file.
-o output file Returns a fasta file with only the selected sequences from the original FASTA sequence file.

Your output file - ACerv.MSdetected.fasta - is your subsetted fasta

1,000 c/L

All ** proteins

Up vs Down regulated

50,000 c/L

All ** proteins

Up vs Down regulated

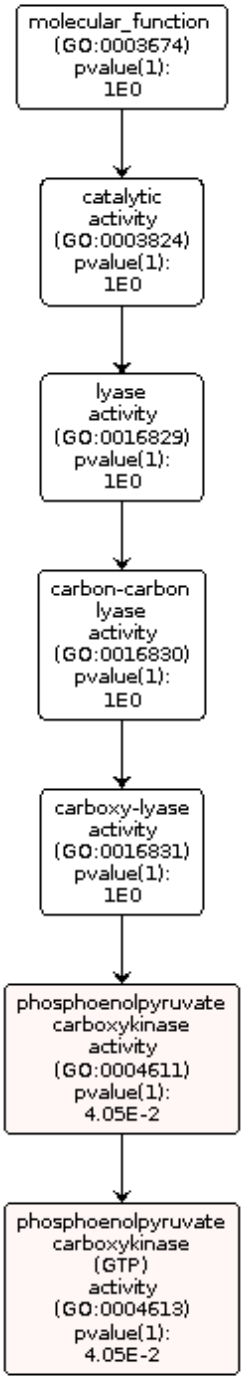
Maldonado Coral CompGO A. cervicornis

website:
https://meta.yeastrc.org/compgo_emma_acerv/pages/goAnalysisForm.jsp

1,000 c/L

All ** proteins

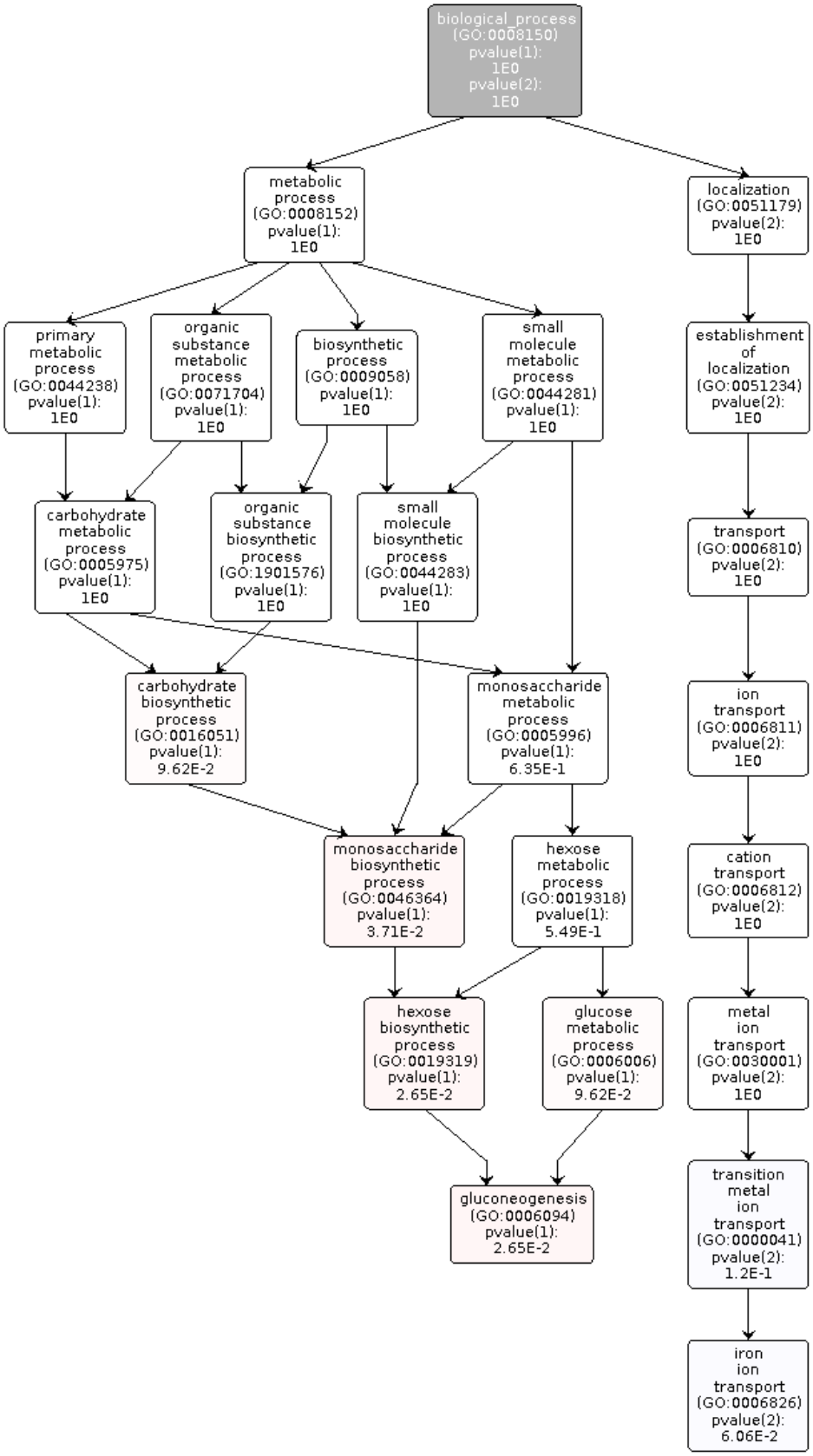
GO Accession	GO Name	GO Aspect	P-value (1)	Protein List (1)
GO:0004611	phosphoenolpyruvate carboxykinase activity	molecular_function	4.05E-02	FUN_010194-T1,FUN_010193-T1
GO:0004613	phosphoenolpyruvate carboxykinase (GTP) activity	molecular_function	4.05E-02	FUN_010194-T1,FUN_010193-T1



Used in gluconeogenesis (generating glucose)

Up vs Down regulated (+ vs - Log fold change)

GO Accession	GO Name	GO Aspect	P-value (1)	P-value (2)	Protein List (1)	Protein List (2)
GO:0019319	hexose biosynthetic process	biological_process	2.65E-02		FUN_010194-T1,FUN_010193-T1	
GO:0006094	gluconeogenesis	biological_process	2.65E-02		FUN_010194-T1,FUN_010193-T1	
GO:0046364	monosaccharide biosynthetic process	biological_process	3.71E-02		FUN_010194-T1,FUN_010193-T1	
GO:0006826	iron ion transport	biological_process		6.06E-02		FUN_002063-T1,FUN_002065-T1
GO:0016051	carbohydrate biosynthetic process	biological_process	9.62E-02		FUN_010194-T1,FUN_010193-T1	
GO:0006006	glucose metabolic process	biological_process	9.62E-02		FUN_010194-T1,FUN_010193-T1	


biological_process
(GO:0008150)
pvalue(1):
1E0
pvalue(2):
1E0
metabolic
process
(GO:0008152)
pvalue(1):
1E0
localization
(GO:0051179)
pvalue(2):
1E0
primary
metabolic
process
(GO:0044238)
pvalue(1):
1E0
organic
substance
metabolic
process
(GO:0071704)
pvalue(1):
1E0
biosynthetic
process
(GO:0009058)
pvalue(1):
1E0
small
molecule
metabolic
process
(GO:0044281)
pvalue(1):
1E0
establishment
of
localization
(GO:0051234)
pvalue(2):
1E0
carbohydrate
metabolic
process
(GO:0005975)
pvalue(1):
1E0
organic
substance
biosynthetic
process
(GO:1901576)
pvalue(1):
1E0
small
molecule
biosynthetic
process
(GO:0044283)
pvalue(1):
1E0
transport
(GO:0006810)
pvalue(2):
1E0
carbohydrate
biosynthetic
process
(GO:0016051)
pvalue(1):
9.62E-2
monosaccharide
metabolic
process
(GO:0005996)
pvalue(1):
6.35E-1
ion
transport
(GO:0006811)
pvalue(2):
1E0
monosaccharide
biosynthetic
process
(GO:0046364)
pvalue(1):
3.71E-2
hexose
metabolic
process
(GO:0019318)
pvalue(1):
5.49E-1
cation
transport
(GO:0006812)
pvalue(2):
1E0
hexose
biosynthetic
process
(GO:0019319)
pvalue(1):
2.65E-2
glucose
metabolic
process
(GO:0006006)
pvalue(1):
9.62E-2
metal
ion
transport
(GO:0030001)
pvalue(2):
1E0
gluconeogenesis
(GO:0006094)
pvalue(1):
2.65E-2
transition
metal
ion
transport
(GO:0000041)
pvalue(2):
1.2E-1
iron
ion
transport
(GO:0006826)
pvalue(2):
6.06E-2

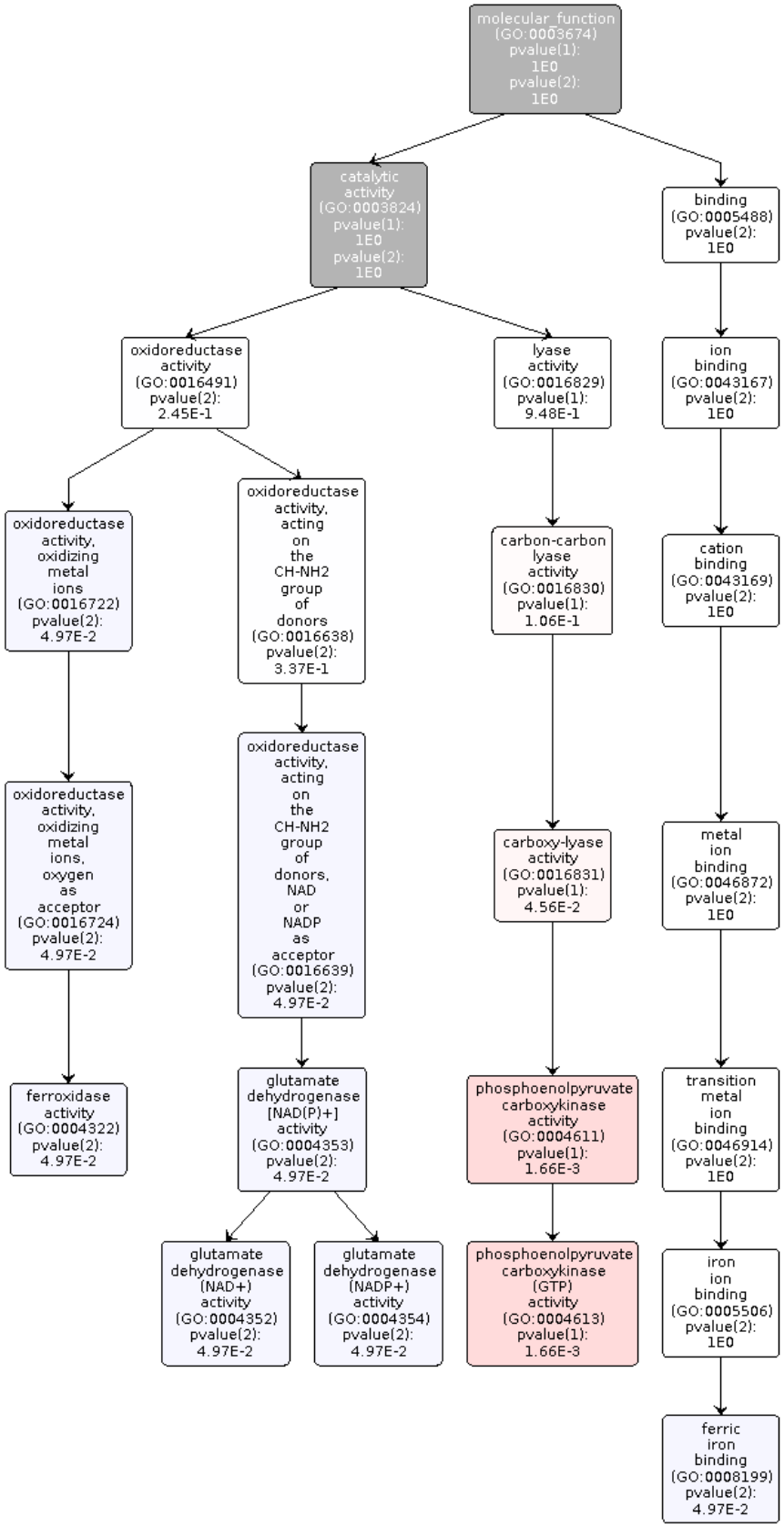
Gluconeogenesis: Creating glucose by breaking down lipids/proteins
Iron ion transport: Directed movement of iron (Fe) ions into, out of or within a cell, or between cells, by means of some agent such as a transporter or pore

---

Up vs Down regulated (+ vs - Log fold change)

GO Accession	GO Name	GO Aspect	P-value (1)	P-value (2)	Protein List (1)	Protein List (2)
GO:0004611	phosphoenolpyruvate carboxykinase activity	molecular_function	1.66E-03		FUN_010194-T1,FUN_010193-T1	
GO:0004613	phosphoenolpyruvate carboxykinase (GTP) activity	molecular_function	1.66E-03		FUN_010194-T1,FUN_010193-T1	
GO:0016831	carboxy-lyase activity	molecular_function	4.56E-02		FUN_010194-T1,FUN_010193-T1	
GO:0008199	ferric iron binding	molecular_function		4.97E-02		FUN_002063-T1,FUN_002065-T1
GO:0004322	ferroxidase activity	molecular_function		4.97E-02		FUN_002063-T1,FUN_002065-T1

GO:0004352	glutamate dehydrogenase (NAD+) activity	molecular_function		4.97E-02		FUN_015848-T1,FUN_027308-T1
GO:0004353	glutamate dehydrogenase [NAD(P)+] activity	molecular_function		4.97E-02		FUN_015848-T1,FUN_027308-T1
GO:0004354	glutamate dehydrogenase (NADP+) activity	molecular_function		4.97E-02		FUN_015848-T1,FUN_027308-T1
GO:0016722	oxidoreductase activity, oxidizing metal ions	molecular_function		4.97E-02		FUN_002063-T1,FUN_002065-T1
GO:0016724	oxidoreductase activity, oxidizing metal ions, oxygen as acceptor	molecular_function		4.97E-02		FUN_002063-T1,FUN_002065-T1
GO:0016639	oxidoreductase activity, acting on the CH-NH2 group of donors, NAD or NADP as acceptor	molecular_function		4.97E-02		FUN_015848-T1,FUN_027308-T1

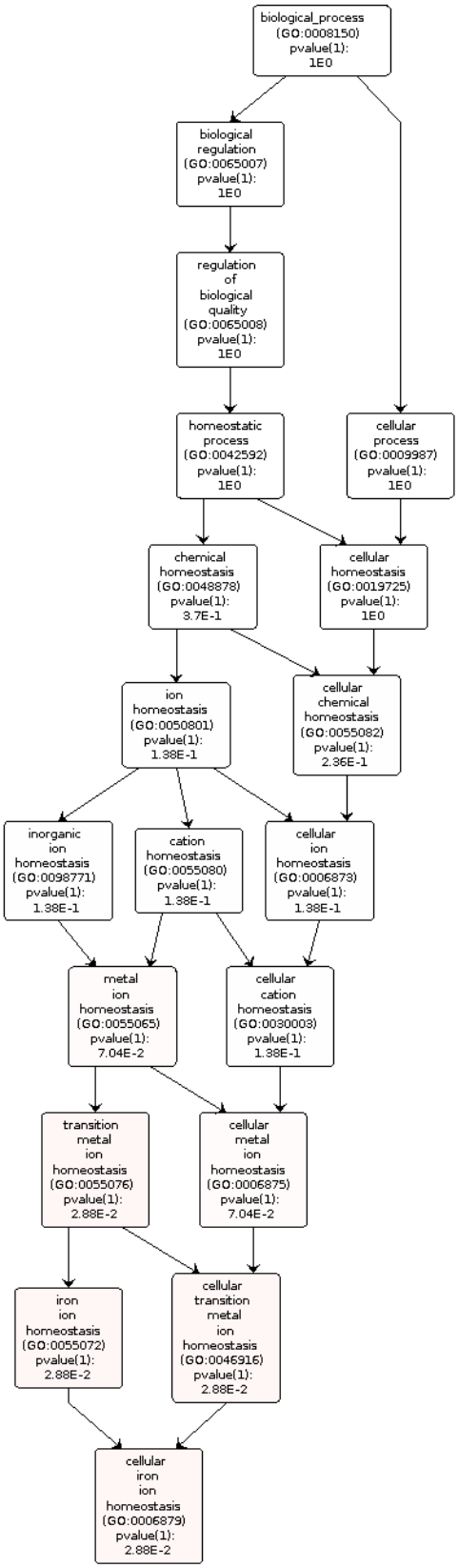

molecular_function (GO:0003674) pvalue(1): 1E0 pvalue(2): 1E0
catalytic activity (GO:0003824) pvalue(1): 1E0 pvalue(2): 1E0
binding (GO:0005488) pvalue(2): 1E0
oxidoreductase activity (GO:0016491) pvalue(2): 2.45E-1
lyase activity (GO:0016829) pvalue(1): 9.48E-1
ion binding (GO:0043167) pvalue(2): 1E0
oxidoreductase activity, oxidizing metal ions (GO:0016722) pvalue(2): 4.97E-2
oxidoreductase activity, acting on the CH-NH2 group of donors (GO:0016638) pvalue(2): 3.37E-1
carbon-carbon lyase activity (GO:0016830) pvalue(1): 1.06E-1
cation binding (GO:0043169) pvalue(2): 1E0
oxidoreductase activity, oxidizing metal ions, oxygen as acceptor (GO:0016724) pvalue(2): 4.97E-2
oxidoreductase activity, acting on the CH-NH2 group of donors, NAD or NADP as acceptor (GO:0016639) pvalue(2): 4.97E-2
carboxy-lyase activity (GO:0016831) pvalue(1): 4.56E-2
metal ion binding (GO:0046872) pvalue(2): 1E0
ferroxidase activity (GO:0004322) pvalue(2): 4.97E-2
glutamate dehydrogenase [NAD(P)+] activity (GO:0004353) pvalue(2): 4.97E-2
phosphoenolpyruvate carboxykinase activity (GO:0004611) pvalue(1): 1.66E-3
transition metal ion binding (GO:0046914) pvalue(2): 1E0
glutamate dehydrogenase (NAD+) activity (GO:0004352) pvalue(2): 4.97E-2
glutamate dehydrogenase (NADP+) activity (GO:0004354) pvalue(2): 4.97E-2
phosphoenolpyruvate carboxykinase (GTP) activity (GO:0004613) pvalue(1): 1.66E-3
iron ion binding (GO:0005506) pvalue(2): 1E0
ferric iron binding (GO:0008199) pvalue(2): 4.97E-2

Ferroxidase activity: Helps with cellular uptake of iron

50,000 c/L

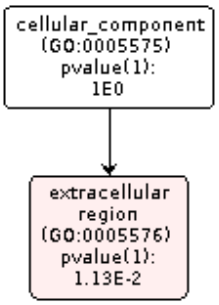
All ** proteins

GO Accession	GO Name	GO Aspect	P-value (1)	Protein List (1)
GO:0055076	transition metal ion homeostasis	biological_process	2.88E-02	FUN_002063-T1,FUN_002065-T1,FUN_009194-T1
GO:0046916	cellular transition metal ion homeostasis	biological_process	2.88E-02	FUN_002063-T1,FUN_002065-T1,FUN_009194-T1
GO:0055072	iron ion homeostasis	biological_process	2.88E-02	FUN_002063-T1,FUN_002065-T1,FUN_009194-T1
GO:0006879	cellular iron ion homeostasis	biological_process	2.88E-02	FUN_002063-T1,FUN_002065-T1,FUN_009194-T1
GO:0055065	metal ion homeostasis	biological_process	7.04E-02	FUN_002063-T1,FUN_002065-T1,FUN_009194-T1
GO:0006875	cellular metal ion homeostasis	biological_process	7.04E-02	FUN_002063-T1,FUN_002065-T1,FUN_009194-T1


biological_process
(GO:0008150)
pvalue(1):
1E0
biological
regulation
(GO:0065007)
pvalue(1):
1E0
regulation
of
biological
quality
(GO:0065008)
pvalue(1):
1E0
homeostatic
process
(GO:0042592)
pvalue(1):
1E0
cellular
process
(GO:0009987)
pvalue(1):
1E0
chemical
homeostasis
(GO:0048878)
pvalue(1):
3.7E-1
cellular
homeostasis
(GO:0019725)
pvalue(1):
1E0
ion
homeostasis
(GO:0050801)
pvalue(1):
1.38E-1
cellular
chemical
homeostasis
(GO:0055082)
pvalue(1):
2.36E-1
inorganic
ion
homeostasis
(GO:0098771)
pvalue(1):
1.38E-1
cation
homeostasis
(GO:0055080)
pvalue(1):
1.38E-1
cellular
ion
homeostasis
(GO:0006873)
pvalue(1):
1.38E-1
metal
ion
homeostasis
(GO:0055065)
pvalue(1):
7.04E-2
cellular
cation
homeostasis
(GO:0030003)
pvalue(1):
1.38E-1
transition
metal
ion
homeostasis
(GO:0055076)
pvalue(1):
2.88E-2
cellular
metal
ion
homeostasis
(GO:0006875)
pvalue(1):
7.04E-2
iron
ion
homeostasis
(GO:0055072)
pvalue(1):
2.88E-2
cellular
transition
metal
ion
homeostasis
(GO:0046916)
pvalue(1):
2.88E-2
cellular
iron
ion
homeostasis
(GO:0006879)
pvalue(1):
2.88E-2

Up vs Down regulated (+ vs - Log fold change)

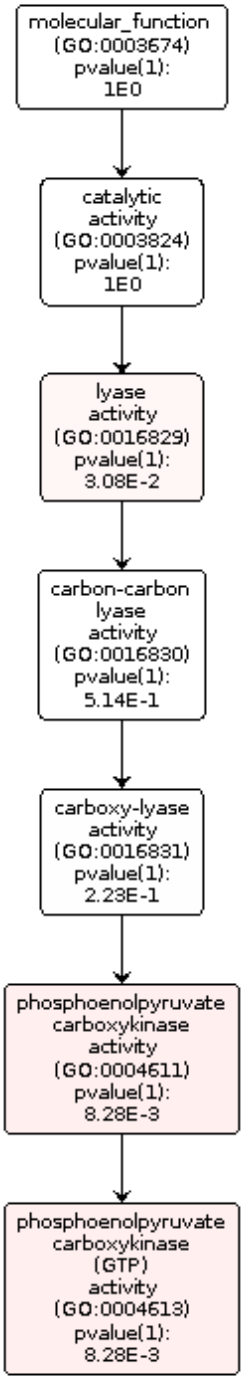
GO Accession	GO Name	GO Aspect	P-value (1)	P-value (2)	Protein List (1)	Protein List (2)
GO:0005576	extracellular region	cellular_component	1.13E-02	1.00E+00	FUN_017330-T1,FUN_015125-T1,FUN_015124-T1,FUN_006334-T1,FUN_020709-T1	FUN_028246-T1



Up vs Down regulated (+ vs - Log fold change)

GO Accession	GO Name	GO Aspect	P-value (1)	Protein List (1)
GO:0004611	phosphoenolpyruvate carboxykinase activity	molecular_function	8.28E-03	FUN_010194-T1,FUN_010193-T1
GO:0004613	phosphoenolpyruvate carboxykinase (GTP) activity	molecular_function	8.28E-03	FUN_010194-T1,FUN_010193-T1

GO:0016829	lyase activity	molecular_function	3.08E-02	FUN_017330-T1,FUN_010194-T1,FUN_010193-T1,FUN_009194-T1



S. sidersatrea Tephra Search

Directory: /net/nunn/vol1/home/rschauer/2021_Dec_10_Side
Comet.params Database:
/net/nunn/vol1/emmats/databases/davies_Ssid_contam_symb.fasta
Files:
2021_Dec_10_Maldonado_CORAL_22.raw
2021_Dec_10_Maldonado_CORAL_23.raw
2021_Dec_10_Maldonado_CORAL_24.raw
2021_Dec_10_Maldonado_CORAL_25.raw
2021_Dec_10_Maldonado_CORAL_26.raw
2021_Dec_10_Maldonado_CORAL_27.raw
2021_Dec_10_Maldonado_CORAL_36.raw
2021_Dec_10_Maldonado_CORAL_37.raw
2021_Dec_10_Maldonado_CORAL_38.raw
2021_Dec_10_Maldonado_CORAL_39.raw
2021_Dec_10_Maldonado_CORAL_43.raw
2021_Dec_10_Maldonado_CORAL_45.raw

```
# Name of protXML file corresponding to merged/combined results
combinedFile=/net/nunn/vol1/home/rschauer/2021_Dec_10_Side/interact-COMBINED.prot.xml

# The directory that contains the pepXML and protXML files
srcDir=/net/nunn/vol1/home/rschauer/2021_Dec_10_Side

# The name of the file where results will be saved to
outputFile=/net/nunn/vol1/home/rschauer/2021_Dec_10_Side/ABACUS_output.tsv

# The path the the FASTA formatted file used for the original protein search
# Relative paths are allowed
fasta=/net/nunn/vol1/emmats/databases/davies_Ssid_contam_symb.fasta

# The minimum PeptideProphet score the best peptide match of a protein must have
maxIniProbTH=0.99

# The minimum PeptideProphet score a peptide must have in order to be even considered by Abacus
iniProbTH=0.50

# E.P.I: Experimental Peptide-probability Inclusion threshold
# If a protein does not contain at least one peptide exceeding this PeptideProphet score, none of the
# peptide evidence for this protein will be considered. This is applied on an experiment by experiment case.
epiTH=0

# The minimum ProteinProphet score a protein group must have in the COMBINED file
minCombinedFilePw=0.92

# If true, Abacus will write ALL protein IDs belonging to a group in the COMBINED file
# Protein IDs starting with ':::' are additional identifiers from the same protein group in
# the COMBINED file. The representative protein for the group does not start with ':::'
verboseResults=false

# The keep the HyperSQL database files that are created after the program is done
keepDB=false

# Should the peptide weights be recalculated in the individual experiment XML files.
# Useful for peptides that are highly degenerate within a single protein groups
recalcPepWts=false

# Spectral count data will be reported in NSAF format.
# NSAF = _N_ormalized _S_pectral _A_bundance _F_actor
# For a detailed explanation of this method refer to this pubmed link:
# http://www.ncbi.nlm.nih.gov/pubmed/20166708
# Abacus reports NSAF values multiplied by a scaling factor. This is done to
# control for numeric underflow (ie: really small numbers). The scaling factor
# that is used is called the NSAF_FACTOR and is reported during runtime in
# case you would like to rescale your data.
asNSAF=true

# If you are using decoy proteins in your searches, specify the first few
# characters of the label indicating decoy proteins here
decoyTag=DECOY_

# Output format that will be produced by this parameter file
output=Default
```

S. sidersatrea Coral Qspec

Making qspec input file

Want a qspec input file with protein id, protein length, and each sample's spectral counts in the end.

Steps:

Read in ABACUS output file

Make speccounts df with spec count columns (NUMSPECTOT)
Remove column ALL_NUMSPECSTOT
Add PROTID column making speccounts2 df
Subset only proteins that have at least 2 unique peptides in twopeps df
Add PROTLEN column making qspec.len final file and export as .csv

Siderastrea coral qspec:

Qspec_Maldonado_Coral_Side.csv 133 kB

contams have been removed

Control vs 1K

CORAL_22, CORAL_39, CORAL_45 vs CORAL_23, CORAL_27, CORAL_38

```
tephra /net/nunn/vol1/home/rschauer/2021_Dec_10_Side/qspec
> qspec-param Sside_qspec_Controlvs1K.txt 2000 10000 1
2335 Proteins and 6 Experiments
Burn-in: 1000   2000    ....done.
Iteration: 1000 2000    3000    4000    5000    6000    7000    8000    9000
10000   ....done.
Run time: 76.990000 seconds

tephra /net/nunn/vol1/home/rschauer/2021_Dec_10_Side/qspec
> getfdr Sside_qspec_Controlvs1K.txt_qspec
Data has 2335 rows and 10 columns
Model Fitting
Searching for rescaling factor
Found re-scaling factor: 0.29048849
The estimate of pi(DE) is 0.356778
```

Analyzing .txt_qspec_fdr file

54 proteins meet LFC and zstat thresholds

Control vs 50K

CORAL_22, CORAL_39, CORAL_45 vs CORAL_24, CORAL_26, CORAL_43

```
tephra /net/nunn/vol1/home/rschauer/2021_Dec_10_Side/qspec
> qspec-param Sside_qspec_Controlvs50K.txt 2000 10000 1
2326 Proteins and 6 Experiments
Burn-in: 1000   2000    ....done.
```

```
Iteration: 1000 2000    3000    4000    5000    6000    7000    8000    9000
10000   ....done.
Run time: 77.130000 seconds

tephra /net/nunn/vol1/home/rschauer/2021_Dec_10_Side/qspec
> getfdr Sside_qspec_Controlvs50K.txt_qspec
Data has 2326 rows and 10 columns
Model Fitting
Searching for rescaling factor
Found re-scaling factor: 0.37346428
The estimate of pi(DE) is 0.312875
```

Analyzing .txt_qspec_fdr file

27 proteins meet LFC and zstat thresholds

Control vs 100K

CORAL_22, CORAL_39, CORAL_45 vs CORAL_25, CORAL_37, CORAL_36

```
tephra /net/nunn/vol1/home/rschauer/2021_Dec_10_Side/qspec
> qspec-param Sside_qspec_Controlvs100K.txt 2000 10000 1
2342 Proteins and 6 Experiments
Burn-in: 1000   2000    ....done.
Iteration: 1000 2000    3000    4000    5000    6000    7000    8000    9000
10000   ....done.
Run time: 77.090000 seconds

tephra /net/nunn/vol1/home/rschauer/2021_Dec_10_Side/qspec
> getfdr Sside_qspec_Controlvs100K.txt_qspec
Data has 2342 rows and 10 columns
Model Fitting
Searching for rescaling factor
Found re-scaling factor: 0.35516081
The estimate of pi(DE) is 0.315841
```

Analyzing .txt_qspec_fdr file

32 proteins meet LFC and zstat thresholds

Converting Maldonado .pep.xmls to limelight

A. cervicornis

Directory with A. cerv Maldonado limelight files
/net/nunn/vol1/home/rschauer/2021_Dec_10_Cerv/limelight
java -jar cometTPP2LimelightXML.jar -c /net/nunn/vol1/home/rschauer/2021_Dec_10_Cerv/comet.params -f /net/nunn/vol1/databases/Acropora_cervicornis.proteins.symb.contam.fasta -p /net/nunn/vol1/home/rschauer/2021_Dec_10_Cerv/interact-2021_Dec_10_Maldonado_CORAL_29.pep.xml -v -o /net/nunn/vol1/home/rschauer/2021_Dec_10_Cerv/limelight/CORAL_29_limelight.xml

S. sidersatrea

Directory with S. side Maldonado limelight files
/net/nunn/vol1/home/rschauer/2021_Dec_10_Adig/limelight
java -jar cometTPP2LimelightXML.jar -c /net/nunn/vol1/home/rschauer/2021_Dec_10_Side/comet.params -f /net/nunn/vol1/emmats/databases/davies_Ssid_contam_symb.fasta -p /net/nunn/vol1/home/rschauer/2021_Dec_10_Side/interact-2021_Dec_10_Maldonado_CORAL_22.pep.xml -v -o /net/nunn/vol1/home/rschauer/2021_Dec_10_Side/limelight/CORAL_22_limelight.xml

General limelight file conversion instructions:

In home directory run:

```
wget https://github.com/yeastrc/limelight-import-comet-tpp/releases/download/v2.6.0/cometTPP2LimelightXML.jar
```

You should now have cometTPP2LimelightXML.jar in your home directory.

Run

```
java -jar cometTPP2LimelightXML.jar -h
```

to see command line parameters and ensure it is working

To convert data:

```
java -jar cometTPP2LimelightXML.jar -c /path/to/comet.params -f /path/to/file.fasta -p /path/to/pep.xml -v -o /path/to/save/limelight.xml
```

Maldonado Coral BLAST

https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome

Acropora_cervicornis.proteins_fas... 14 MB

S_sidersatrea.proteins_fasta.txt 75 MB

104 proteins

Acerv_BLAST.xlsx

83 proteins

Sside_BLAST.xlsx

Qspec Protocol

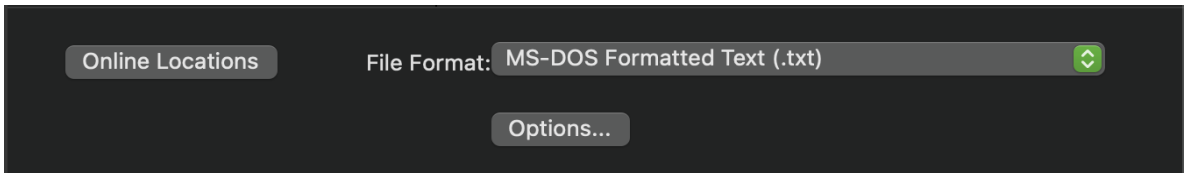
Making input file

Columns to have for qspec: PROTID, PROTLEN, Treatment 1 NUMSPECOT columns, Treatment 2 NUMSPECOT columns

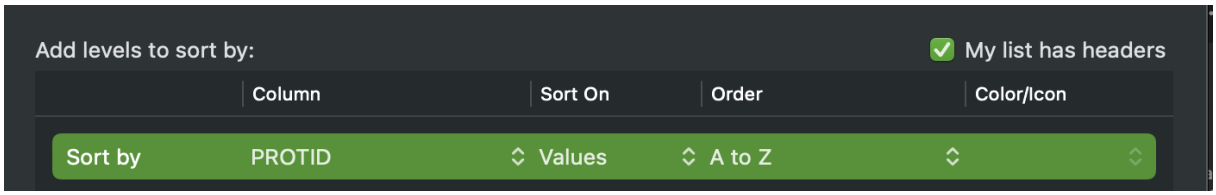
Making qspec file.R 1 kB

Formatting the .csv file:

1. Clean up column names for legibility
2. Choose two treatments to compare (control vs something) and save as a MS-DOS Formatted Text file with them in the name (no spaces)



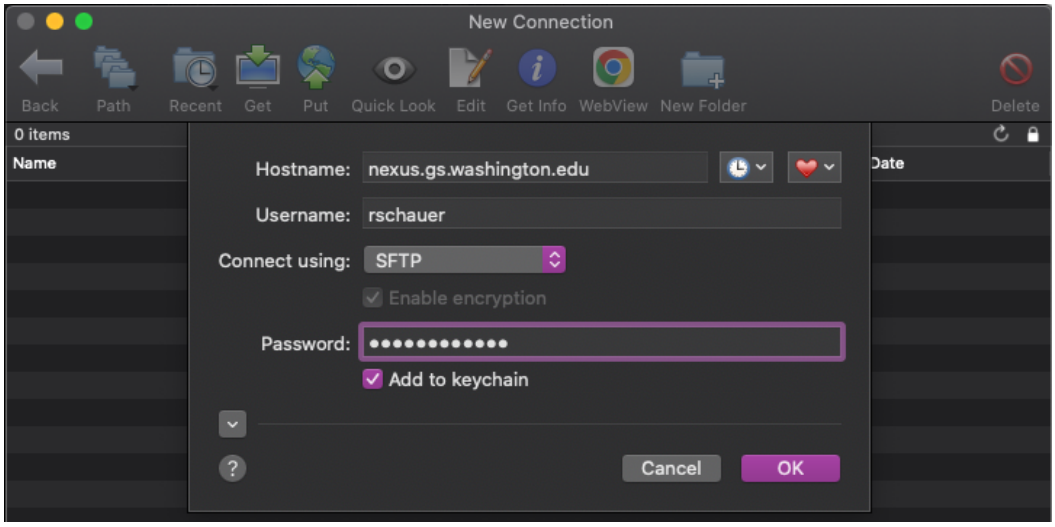
3. Delete any other treatment columns (compare in separate file) and make the column headers for one treatment "0" and the other "1"
4. Data > Sort & Filter > Sort by PROTID



5. Delete rows that are contaminates (will have HUMAN/BOVIN/SHEEP/PIG)

2535	K2M1_SHEEP	109	14	6	0	0	0	0	20
2536	PRDX1_HUMAN	199	5	6	4	5	4	5	29
2537	TRYP_PIG	231	25	22	15	31	28	17	138

6. Add a column at the end and sum spec count values, drag down and autofill for all proteins
7. Data > Sort & Filter > Sort by sums column
8. Delete rows where sum = 0
9. Delete sums column
10. Login to Fetch and upload file to project directory (put in separate "qspec" folder)



Running qspec on the cluster:

ssh rschauer@nexus.gs.washington.edu

ssh tephra

(Optional) check qspec parameters

```
> qspec-param
usage: qspec-param <matrixData> <nburnin> <niter> <normalize?(0/1)>
```

1. Run qspec, parameters are always: 2000 10000 1

```
> qspec-param [FILE NAME].txt 2000 10000 1
#### Proteins and # Experiments
Burn-in: 1000   2000    ....done.
Iteration: 1000 2000    3000    4000    5000    6000    7000    8000    9000
10000   ....done.
Run time: ##.# seconds
```

2. Add false discovery rate using new .txt_qspec file

```
> getfdr [FILE NAME].txt_qspec
Data has #### rows and ## columns
Model Fitting
Searching for rescaling factor
Found re-scaling factor: #.##
The estimate of pi(DE) is #.##
```

3. Download new .txt_qspec_fdr file using Fetch

Analyzing .txt_qspec_fdr file

1. Open file in Excel
2. Add two columns at the end and name them LFC and z stat
3. In LFC column mark proteins whose log fold change is over 0.5 using formula =IF(ABS(##)>0.5, "*",)
    - Enter name of first cell in LogFoldChange column for ##
4. In z stat column mark proteins whose z statistic is over 2 using formula =IF(ABS(##)>2, "*",)
    - Enter name of first cell in Zstatistic column for ##
5. Drag down and autofill both columns for all proteins
6. Data > Sort & Filter > Sort by LFC then by z stat
    - Order is largest to smallest to have *s at top
7. Note how many proteins have * in both columns

A.Cervicornis Coral Qspec

Making qspec input file

Want a qspec input file with protein id, protein length, and each sample's spectral counts in the end.

Steps:

- Read in ABACUS output file
- Make speccounts df with spec count columns (NUMSPECTOT)
- Remove column ALL_NUMSPECSTOT
- Add PROTID column making speccounts2 df
- Subset only proteins that have at least 2 unique peptides in twopeps df
- Add PROTLEN column making qspec.len final file and export as .csv

Cervicornis coral qspec

Qspec_Maldonado_Coral_Cerv.csv 96 kB

contams have been removed

Control vs 1K

CORAL_29, CORAL_30, CORAL_40 vs CORAL_31, CORAL_32, CORAL_44

Running qspec on the cluster

```
tephra /net/nunn/vol1/home/rschauer/2021_Dec_10_Cerv/qspec
> qspec-param Acerv_qspec_Controlvs1K.txt 2000 10000 1
2499 Proteins and 6 Experiments
Burn-in: 1000   2000    ....done.
Iteration: 1000 2000    3000    4000    5000    6000    7000    8000    9000
10000   ....done.
Run time: 85.060000 seconds

tephra /net/nunn/vol1/home/rschauer/2021_Dec_10_Cerv/qspec
> getfdr Acerv_qspec_Controlvs1K.txt_qspec
Data has 2499 rows and 10 columns
Model Fitting
Searching for rescaling factor
Found re-scaling factor: 0.45660975
The estimate of pi(DE) is 0.284501
```

Analyzing .txt_qspec_fdr file

62 proteins meet LFC and zstat thresholds

Acerv_qspec_Controlvs1K.txt_qs... 185 kB

Control vs 50K

CORAL_29, CORAL_30, CORAL_40 vs CORAL_33, CORAL_34, CORAL_41

Running qspec on the cluster

```
tephra /net/nunn/vol1/home/rschauer/2021_Dec_10_Cerv/qspec
> qspec-param Acerv_qspec_Controlvs50K.txt 2000 10000 1
2512 Proteins and 6 Experiments
Burn-in: 1000   2000    ....done.
Iteration: 1000 2000    3000    4000    5000    6000    7000    8000    9000
10000   ....done.
Run time: 92.200000 seconds

tephra /net/nunn/vol1/home/rschauer/2021_Dec_10_Cerv/qspec
> getfdr Acerv_qspec_Controlvs50K.txt_qspec
Data has 2512 rows and 10 columns
Model Fitting
Searching for rescaling factor
Found re-scaling factor: 0.38104712
The estimate of pi(DE) is 0.297375
```

Analyzing .txt_qspec_fdr file

92 proteins meet LFC and zstat thresholds

Acerv_qspec_Controlvs50K.txt_q... 187 kB

**Mike just has proteins from CompGO portal, does not include symbiont proteins

Hela cell filter prep

Batch control, one 20 ug sample per s-trap batch
3/17/23

We want 20 ug protein per sample per batch
Need 8 batches - round up to 10
20 ug x 10 batches = 200 ug protein total

Have ~3 mg protein - resuspend in 1 mL ice cold 1X PBS
= 3 ug/uL protein estimate

200 ug protein / 3 ug/uL = 66 uL total
66 uL over 3 filters = 22 uL cells per filter

Add 1 extra filter

V1: 1 filter

1. thaw cell pellet on ice, put 1X PBS on ice
2. resuspend cell pellet in 1 mL 1X PBS - vortex at medium/low speed
3. take aliquot of 100 uL, transfer to microfuge tube
4. spin original cell suspension 300 RPM, 5 min, 10C - remove liquid- give back to Chris
5. add 22 uL to 4 different PES filters in ziplock baggie
6. freeze filters 30 min at -80C
7. proceed with squishing normally
8. sonicate normal
9. if time, put on speed vac

Rachel noticed that because the filters were dry, the final volume coming off the filters after squishing was only 200 uL total instead of expected 400 uL.
Need to repeat but this time pre-wet filters with 1 mL 1X PBS and remove extra liquid prior to adding cells.

V2: 2 filters

1. thaw cell pellet on ice, put 1X PBS on ice
2. put filters in baggies shiny side up - add 1 mL 1X PBS - remove excess liquid from baggie
3. resuspend cell pellet in 0.9 mL 1X PBS - vortex at medium speed ~5
4. take aliquot of 100 uL, transfer to microfuge tube

5. spin original cell suspension 500 RPM, 5 min, 10C - remove liquid- stored in Rachel's -80 box
6. add 22 uL to 4 different PES filters in ziplock baggie
7. freeze filters 30 min at -80C
8. proceed with squishing normally
9. sonicate normal
10. freeze -80 in Rachel box - also contains standards for running BCA

Salts interact with SDS so the bubbles post squishing wouldn't spin down.
Will repeat by wetting the filters with water instead of PBS, abandon the previous batch of filters.
Color dot stickers weigh 0.005g.

V3: 4 filters - USING THIS METHOD X2 (8 TOTAL FILTERS) DUE TO FIRST BCA A LITTLE LOW

1. thaw cell pellet on ice, put 1X PBS on ice
2. put filters in baggies shiny side up - add 1 mL water - remove excess liquid from baggie
3. resuspend cell pellet in 0.8 mL 1X PBS - vortex at medium speed ~5
4. take aliquot of 200 uL, transfer to microfuge tube
5. spin original cell suspension 500 RPM, 5 min, 10C - remove liquid- stored in Rachel's -80 box
6. add 40 uL to 4 different PES filters in ziplock baggie - due to estimated cell loss from spin downs...need to recalculate expected protein amounts
7. freeze filters 30 min at -80C
8. proceed with squishing normally
9. sonicate normal
10. freeze -80 in Rachel box - also contains standards for running BCA

BCA Protocol

Determining protein concentration of your sample and ultimately how much protein you have. If you think you'll need a lot for s-traps consider diluting your sample.

If you think you have a very concentrated sample, do a quick dilution test with 4 wells: no dilution, 1:1, 1:5, and 1:10; incubate 30 min with reagent and chose the one that is lavender.

Making BSA Standards

Can be kept in -80

Vial	Volume of Diluent (likely 5% SDS buffer) (uL)	Volume & Source of BSA (glass aliquots in BCA box) (uL)	Final BSA Concentration (ug/mL)
A	0	300 of Stock	2000
B	125	375 of Stock	1500
C	325	325 of Stock	1000
D	175	175 of Vial B dilution	750
E	325	325 of Vial C dilution	500
F	325	325 of Vial E dilution	250
G	325	325 of vial F dilution	125
H	400	100 of vial G dilution	25
I	400	0	0 = Blank

Prior to Making Plate

1. Fill out template with your plate layout
    - 96-well plate template.pdf 43 kB
2. CALCULATE 50:1 ratio A:B
    1. # wells you will use * 200 uL = Volume Reagent A (round up to whole mL, like to be ~600 uL over to be safe)

    2. Volume Reagent A / 50 = Volume Reagent B
3. Aliquot Reagent A in a falcon tube, wait to add Reagent B (BCA box above bench)

Make the Plate

When pipetting, press tip to the bottom of the well and only go down to first stop on pipette (make sure to be consistent and do this for all samples)

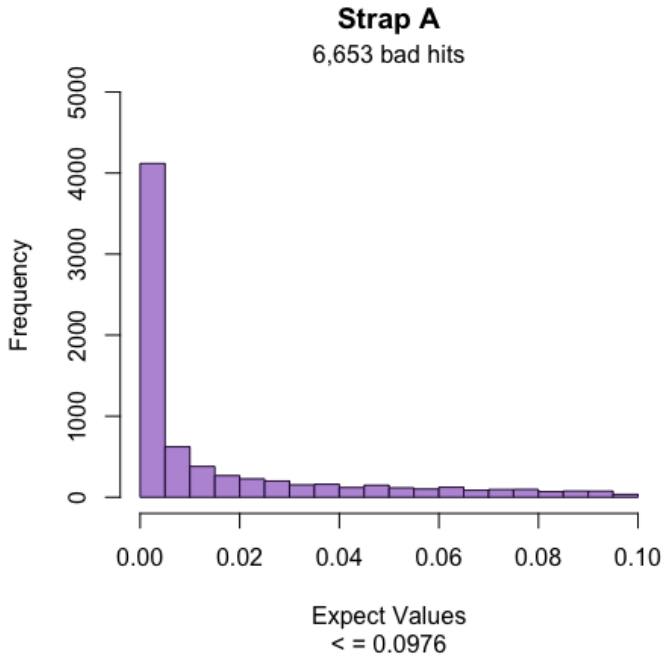
1. Add 10 uL standards (A-I) along top 3 rows of the plate with replicates in columns
2. Add 10 uL samples with replicates if you are doing them
3. Add Reagent B to Reagent A and vortex
4. Add 200 uL Reagent solution to every occupied well (do standards last) and gently shake, check for bubbles

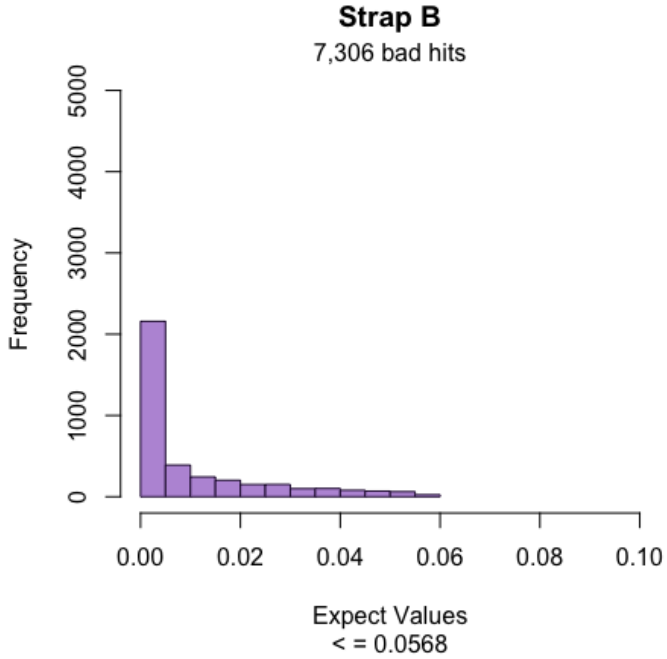
Read the Plate

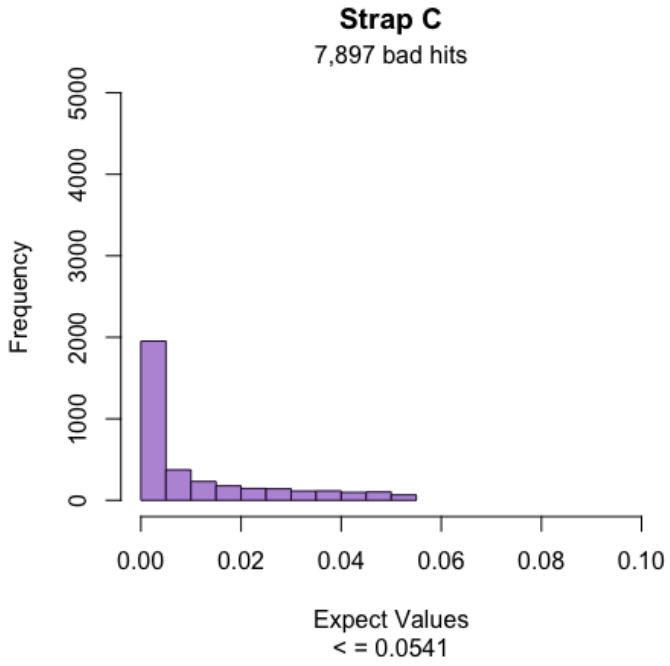
1. Heat plate @ 37°C for 30 min (hallway)
2. Read plate using Varioskan
    1. Turn on machine then log into computer, click icon w/o box in upper left
    2. Click your folder then an old session, immediately save as with new name
    3. Adjust plate layout
    4. Insert plate into machine and run session, take picture of results!
    5. Save report to a USB
3. Fill out BCA Excel sheet to find sample protein concentrations in ug/mL and uL volume needed for 50 ug of protein
    1. Blank values (Standard I) should be around 0.15
4. Aliquot 50 ug protein for each sample in 1.5 mL tubes
5. Add SDS buffer so each sample has 40 uL total volume
6. Freeze in -80

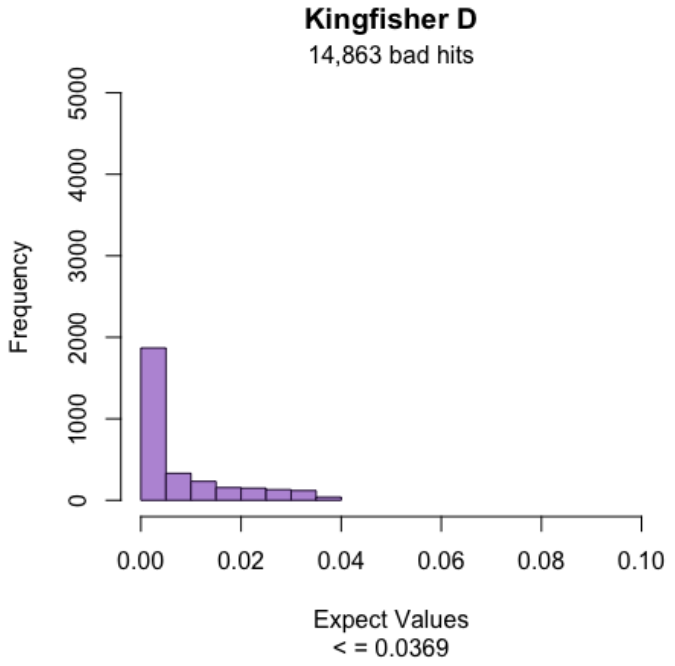
HAB Methods Test Histograms

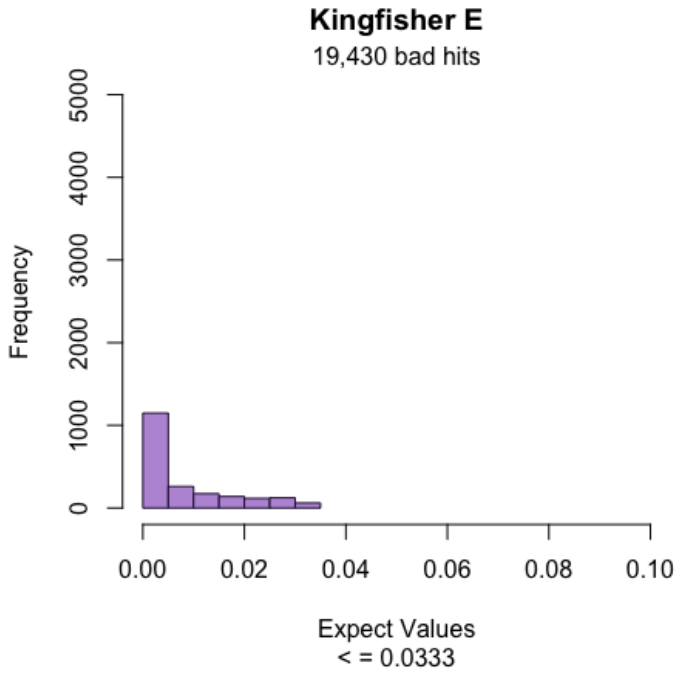
Histograms of expect values from .pep.xml files at or below e score threshold @ FDR 5% calculated by Jimmy

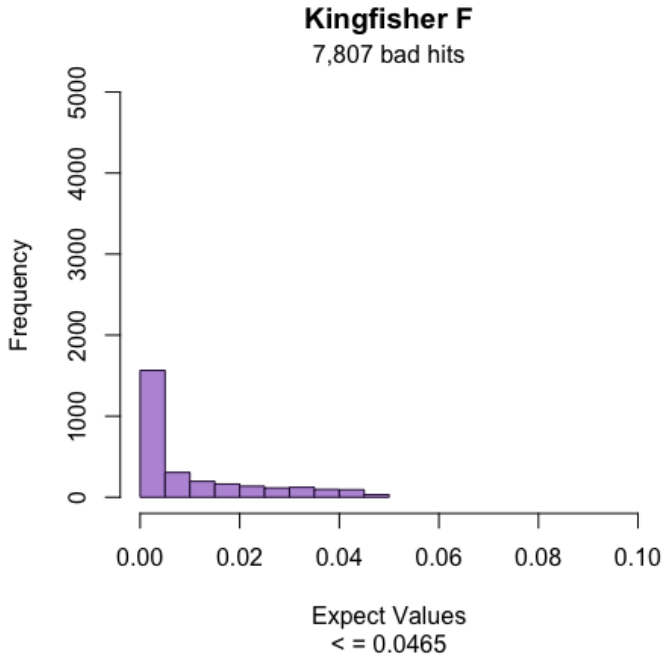

Strap A
6,653 bad hits
Frequency
0
1000
2000
3000
4000
5000
0.00
0.02
0.04
0.06
0.08
0.10
Expect Values
< = 0.0976


Strap B
7,306 bad hits
Frequency
0
1000
2000
3000
4000
5000
0.00
0.02
0.04
0.06
0.08
0.10
Expect Values
< = 0.0568

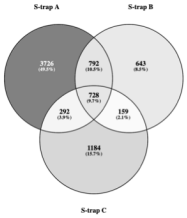

Strap C
7,897 bad hits
Frequency
0
1000
2000
3000
4000
5000
0.00
0.02
0.04
0.06
0.08
0.10
Expect Values
< = 0.0541


Kingfisher D
14,863 bad hits
Frequency
0
1000
2000
3000
4000
5000
0.00
0.02
0.04
0.06
0.08
0.10
Expect Values
< = 0.0369


Kingfisher E
19,430 bad hits
Frequency
0
1000
2000
3000
4000
5000
0.00
0.02
0.04
0.06
0.08
0.10
Expect Values
< = 0.0333

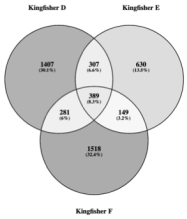


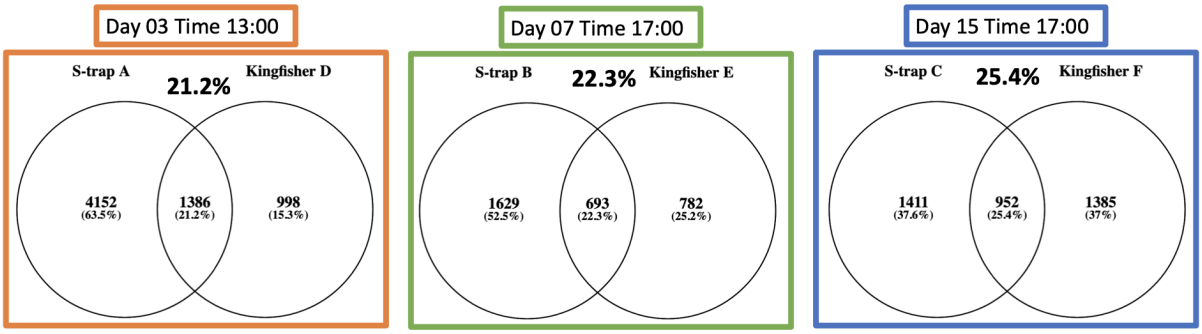
Sample ID	Day	Timepoint	E score threshold FDR 5%	Target PSMs	Decoy PSMs	Unique Peptides
strap A	03	13	0.0976	7629	381	5538
strap B	07	17	0.0568	3866	193	2322
strap C	15	17	0.0541	3632	181	2363
kingfisher D	03	13	0.0369	2908	149	2384
kingfisher E	07	17	0.0333	1988	99	1475
kingfisher F	15	17	0.0465	2892	144	2337



S-trap
26.2%

SP3
24.1%





2021 HAB Filter Processing Squish and BCA

2/23/23

SDS squish

Used highest vol filters

5% SDS Buffer:

For 1,000 uL:

- 50 uL 1M TEAB (cold room)
- 250 uL 20% SDS (bench)
- 2 uL 1 M MgCl2 (bench)
- 688 uL HPLC water (above bench)
- 10 uL 100X HALT protease & phosphatase inhibitors (cold room, come in 100uL tubes)

1. Label and weigh tubes
2. Spray and wipe down all pipettes and gloves with 70% EtOH before each filter
3. Add 100 uL SDS buffer on top of filter in bag
4. Squish 1 min
5. Transfer liquid to eppie tube
6. Add 100 uL HPLC water on top of filter in bag
7. Squish 1 min
8. Transfer liquid to eppie tube
9. Add 100 uL HPLC water on top of filter in bag
10. Press liquid to bottom of bag
11. Transfer liquid to eppie tube
12. Add 100 uL HPLC water on top of filter in bag
13. Press liquid to bottom of bag
14. Transfer liquid to eppie tube

Centrifuge tubes with large amounts of bubbles

6 per person AM & PM, freeze all samples to be speed vacuumed together the next day

Sonicate

1. Wipe off probe with 70% EtOH
2. Sonicate at speed 2.5 for 10 sec then place in ice for 30 sec, 5 times per sample

6-12 sample batches

Speed Vacuum

Settings: no, ccc, 2.00, 1.00

1. Start speed vac, check after ~2 hours
2. Weigh tubes that look to be at or under 100 uL
    1. If less than 0.1 over empty weight, add appropriate vol. of HPLC water
    2. If 0.101-0.110 over empty weight, record how much over & don't add water
    3. If over 0.110 over, put back in speed vacuum
3. Vortex samples
4. Centrifuge @ 4°C and 10,000 rpm for 10 min
5. Freeze in -80

BCA Assay

3/9/23

1. Thawed 66 samples on ice
2. Vortexed
3. Centrifuged @ 4°C and 10,000 rpm for 10 min
4. Pipetted 10 uL supernatant into well (one per sample)
5. Rachel did 2 rows standards, Miranda did 1
6. Determined that SDS was precipitating out due to ice lowering temp, will not thaw on ice next time and spin at room temp
7. Added 20 mL Reagent A to 400 uL Reagent B, vortexed
8. Added 200 uL Reagent solution to every well going in rows starting with A1, disposing of pipette tip after each row
9. Incubated in Varioskan @ 37°C for 30 min then read plate
10. Added 10 uL HPLC water back to all samples, vortexed

3/10/23

1. Thawed 63 samples on bench (all from previous day)
2. Vortexed
3. Centrifuged @ room temp and 10,000 rpm for 10 min
4. Pipetted 10 uL supernatant into well (one per sample)
5. Added three wells of nanopure water
6. Added 20 mL Reagent A to 400 uL Reagent B, vortexed

7. Added 200 uL Reagent solution to every well going in rows, ending with standards, disposing of pipette tip after each row
8. Incubated in Varioskan @ 37°C for 30 min then read plate
    1. Will incubate on warm plate instead going forward since Varioskan does not like being left on this long

BCA_031023_HAB_samples_1.xlsx 32 kB

1. Thawed remaining 43 samples on bench (3 from previous day)
2. Vortexed
3. Centrifuged @ room temp and 10,000g for 10 min
4. Pipetted 10 uL supernatant into well (one per sample)
5. Added three wells of nanopure water
6. Added 15.5 mL Reagent A to 310 uL Reagent B, vortexed
7. Added 200 uL Reagent solution to every well going in rows, ending with standards, disposing of pipette tip after each row
8. Incubated in Varioskan @ 37°C for 30 min then read plate
    1. Will incubate on warm plate instead going forward since Varioskan does not like being left on this long

BCA_031023_HAB_samples_2.xlsx 30 kB

3/15/23
Reprocessing 9 timepoints: 1121, 1401, 1405, 1409, 1413, 1417, 1509, 1513, 1521 due to low protein
Only one 1921 filter left, no 1517 filters left (used for methods testing)

Sample Name	Protein lysate vol. needed for 50 ug S-Trap (uL)	Protein lysate vol. needed for 20 ug S-Trap (uL)	Concentration (ug/ul)	Protein in 90ul (ug)
1409BX	463.06	185.22	0.108	
1509B	409.09	163.64	0.122	
1121C	363.23	145.29	0.138	
1401	320.47	128.19	0.156	
1521D	297.86	119.15	0.168	
1517	260.74	104.30	0.192	17.25825
1417C	255.41	102.17	0.196	
1405D	241.20	96.48	0.207	
1921B	239.61	95.85	0.209	18.78024
1513E	217.81	87.12	0.230	
1413D	215.21	86.09	0.232	
1121X	209.10	83.64	0.239	21.52128

3/36/23

1. Thawed 9 samples plus 1517E (C from methods testing)
2. Vortexed
3. Centrifuged @ room temp and 10,000g for 10 min
4. Pipetted 10 uL supernatant into well (one per sample)
5. Added 8 mL Reagent A to 160 uL Reagent B, vortexed
6. Added 200 uL Reagent solution to every well going in rows, ending with standards, disposing of pipette tip after each row
7. Incubated hot plate 37°C for 30 min
8. Read plate in Varioskan

BCA_031623_HAB_rerun_samples... 25 kB

Filters that will need to be combined:

	Sample 1				Sample 2						
Timepoint	#	Con. (ug/ul)	Protein in sample (ug)	Sample vol. (uL)	#	Con. (ug/ul)	Protein in sample (ug)	Sample vol. (uL)	Sample 1 vol to use (uL)	Sample 2 vol to use (uL)	Total vol (uL)
1417	C	0.196	17.62	90	D	0.076	6.84	90	90	31.34	121.34
1409	BX	0.108	9.72	90	B	0.081	7.29	90	90	126.91	216.91
1401	E	0.156	14.664	94	B	0.119	10.71	90	94	44.84	138.84
1521	D	0.168	16.128	96	A	0.16	14.4	90	96	24.20	120.20
1405	D	0.207	18.837	91	B	0.174	15.66	90	91	6.68	97.68
1517	B	0.192	17.472	91	E	0.204	7.70916	37.79	91	12.39	103.39
1921	B	0.209	18.81	90	D	0.137	12.33	90	90	8.69	98.69

Third 1409 sample ran- has enough protein to not be pooled. 1921 will not be pooled since it is a rep timepoint and #B is close to 20 ug protein.

3/21/23
Aliquoting samples for s-traps: bringing non-rep samples up to 90uL with SDS buffer, rep samples up to 140uL with SDS buffer

2021_HAB_Strap_Aliquots.xlsx 21 kB

HAB Methods Tephra Search & Protein Counts

Directory: /net/nunn/vol1/mmudge/2023_02_13_HAB_methodstesting_k.vs.s/MZMLS
Comet.params Database: /net/nunn/vol1/mmudge/2022_HAB_metagenome/nunn_done/megahit3/HAB.megahit2.proteins.fasta
Files:
2023_02_13_HAB_methodstest_strap_A.raw
2023_02_13_HAB_methodstest_strap_B.raw
2023_02_13_HAB_methodstest_strap_C.raw
2023_02_13_HAB_methodstest_kingfisher_D.raw
2023_02_13_HAB_methodstest_kingfisher_E.raw
2023_02_13_HAB_methodstest_kingfisher_F.raw

interact-COMBINED.prot.xml error rates

TPP::Analysis and Models for interact-COMBINED.prot.xml

Models Charts | Learned Models | Sens/Error Tables | Run Options

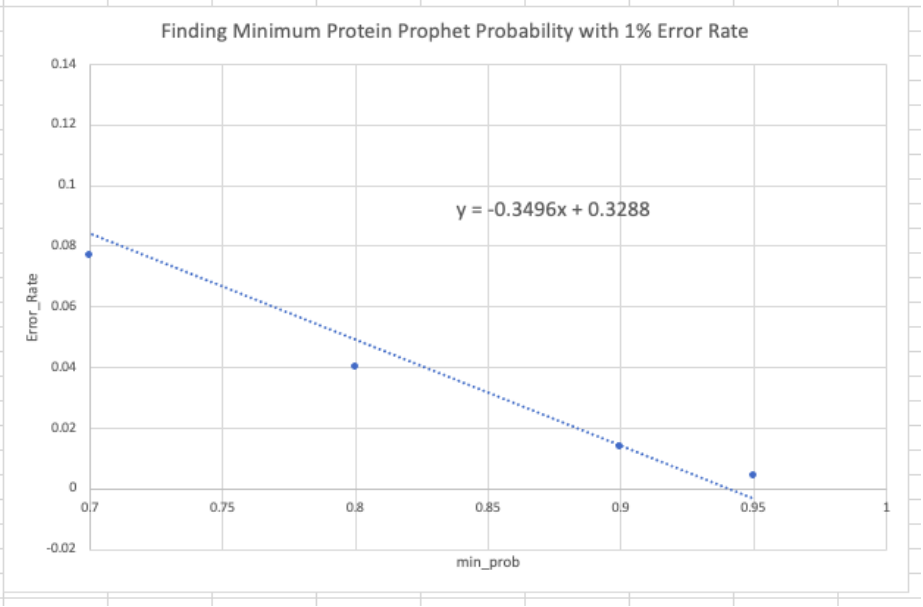
Predicted Sensitivity and Error Rate				
min_prob	Sensitivity	Error_Rate	num_correct	num_incorrect
1.00	0.061	0.000	471	0
0.99	0.339	0.001	2614	3
0.98	0.340	0.001	2627	3
0.97	0.348	0.002	2689	5
0.96	0.360	0.003	2775	8
0.95	0.372	0.004	2872	13
0.90	0.429	0.014	3311	49
0.80	0.515	0.040	3973	165
0.70	0.600	0.077	4631	388
0.60	0.695	0.128	5366	786
0.50	0.783	0.182	6040	1343
0.40	0.867	0.242	6688	2141
0.30	0.939	0.305	7245	3179
0.20	1.000	0.374	7717	4607
0.10	1.000	0.374	7717	4607
0.00	1.000	0.677	7717	16171

Error Table			
Error_Rate	min_prob	num_correct	num_incorrect
0.0000	1.0000	1597	0
0.0000	0.9999	1696	0
0.0000	0.9998	1738	0
0.0000	0.9997	1758	0
0.0000	0.9996	1766	0
0.0000	0.9995	1778	0
0.0000	0.9994	1785	0
0.0000	0.9993	1793	0
0.0000	0.9992	1799	0
0.0000	0.9991	1805	0
0.0000	0.9990	1808	0
0.0000	0.9989	1811	0
0.0000	0.9988	1817	0
0.0000	0.9987	1821	0
0.0000	0.9986	1824	0
0.0000	0.9985	1827	0
0.0000	0.9984	1829	0

Calculating Min Protein Prophet Probability

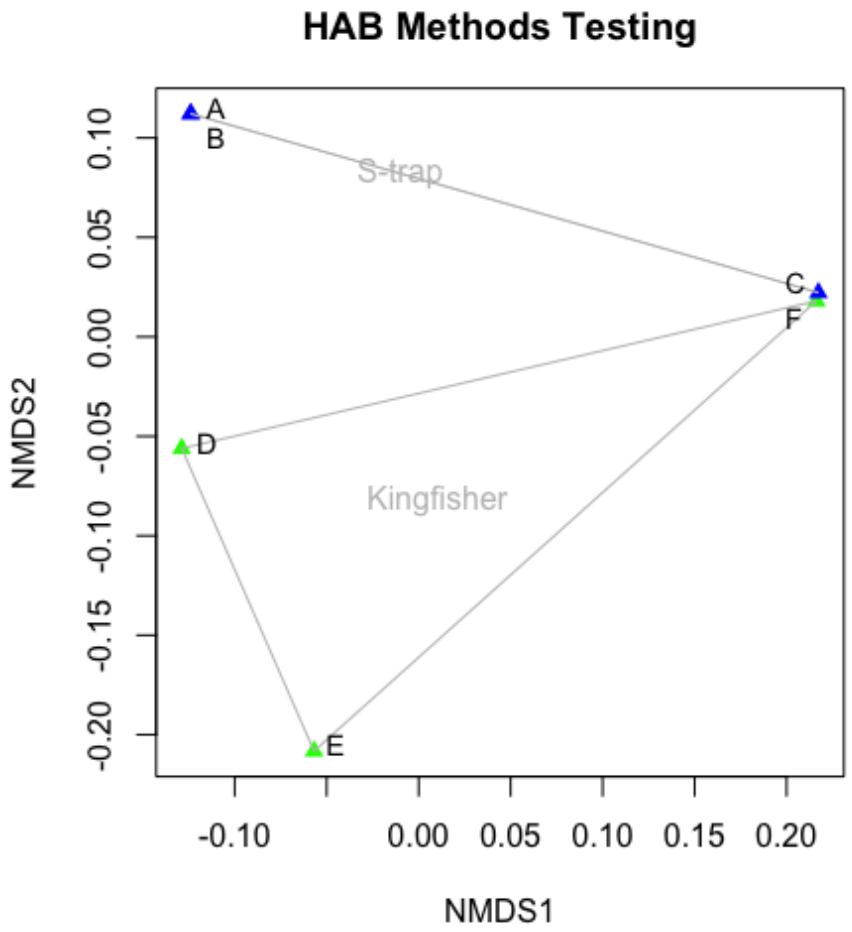
min_prob	Error_Rate
0.95	0.004
0.9	0.014
0.8	0.04
0.7	0.077
0.6	0.128

Lin. Regression	
y	0.01
slope	-0.3496
y-int	0.3288
x	0.9118993



ABACUS Parameter File

```
#
# ABACUS parameter file
# Generated on: 2016Jun30_1144
#

# Name to give the database
dbName=ABACUSDB

# Name of protXML file corresponding to merged/combined results
combinedFile=/net/nunn/vol1/mmudge/2023_02_13_HAB_methodstesting_k.vs.s/MZMLS/interact-COMBINED.prot.xml

# The directory that contains the pepXML and protXML files
srcDir=/net/nunn/vol1/mmudge/2023_02_13_HAB_methodstesting_k.vs.s/MZMLS

# The name of the file where results will be saved to
outputFile=/net/nunn/vol1/mmudge/2023_02_13_HAB_methodstesting_k.vs.s/MZMLS/ABACUS_output.tsv

# The path the the FASTA formatted file used for the original protein search
# Relative paths are allowed
fasta=/net/nunn/vol1/mmudge/2022_HAB_metagenome/nunn_done/megahit3/HAB.megahit2.proteins.fasta

# The minimum PeptideProphet score the best peptide match of a protein must have
maxIniProbTH=0.99

# The minimum PeptideProphet score a peptide must have in order to be even considered by Abacus
iniProbTH=0.50

# E.P.I: Experimental Peptide-probability Inclusion threshold
# If a protein does not contain at least one peptide exceeding this PeptideProphet score, none of the
# peptide evidence for this protein will be considered. This is applied on an experiment by experiment case.
epiTH=0

# The minimum ProteinProphet score a protein group must have in the COMBINED file
minCombinedFilePw=0.91
```



For Kingfisher D and Kingfisher E Repeats

Directory:
/net/nunn/vol1/mmudge/2023_02_13_HAB_methodstesting_k.vs.s/MZXMLS_2ug

Comet.params Database:
/net/nunn/vol1/mmudge/2022_HAB_metagenome/nunn_done/megahit3/HAB.megahit2.proteins.fasta

TPP::Analysis and Models for **interact-COMBINED.prot.xml**

Models Charts | Learned Models | Sens/Error Tables | Run Options

Predicted Sensitivity and Error Rate

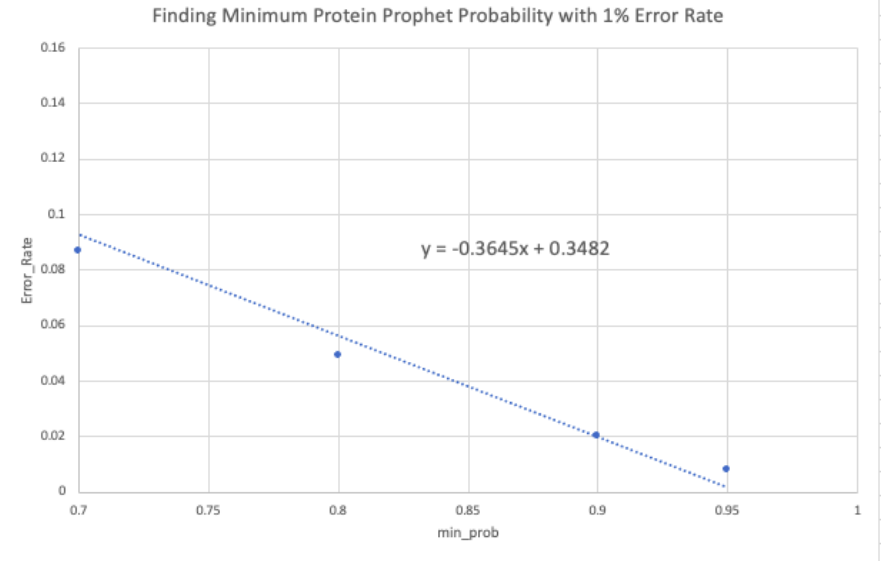
min_prob	Sensitivity	Error_Rate	num_correct	num_incorrect
1.00	0.050	0.000	210	0
0.99	0.272	0.001	1144	1
0.98	0.275	0.001	1158	1
0.97	0.297	0.003	1253	3
0.96	0.311	0.004	1309	5
0.95	0.338	0.008	1425	11
0.90	0.407	0.020	1716	34
0.80	0.510	0.049	2149	110
0.70	0.604	0.087	2546	243
0.60	0.705	0.137	2969	473
0.50	0.796	0.190	3355	789
0.40	0.877	0.246	3694	1207
0.30	0.950	0.308	4003	1780
0.20	1.000	0.364	4213	2415
0.10	1.000	0.364	4213	2415
0.00	1.000	0.553	4213	5223

Error Table

Error_Rate	min_prob	num_correct	num_incorrect
0.0000	1.0000	709	0
0.0000	0.9999	748	0
0.0000	0.9998	768	0
0.0000	0.9997	784	0
0.0000	0.9996	791	0
0.0000	0.9995	794	0
0.0000	0.9993	796	0
0.0000	0.9992	799	0
0.0000	0.9990	800	0
0.0000	0.9988	803	0
0.0000	0.9987	804	0
0.0000	0.9986	807	0
0.0000	0.9984	808	0
0.0000	0.9982	809	0
0.0000	0.9979	810	0
0.0006	0.9978	1123	1
0.0006	0.9972	1124	1

min_prob	Error_Rate
0.95	0.008
0.9	0.02
0.8	0.049
0.7	0.087
0.6	0.137

Lin. Regression	
y	0.01
slope	-0.3645
y-int	0.3482
x	0.9278464



```
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# ABACUS parameter file
# Generated on: 2016Jun30_1144
#

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# Name of protXML file corresponding to merged/combined results
combinedFile=/net/nunn/vol1/mmudge/2023_02_13_HAB_methodstesting_k.vs.s/MZXMLS_2ug/interact-COMBINED.prot.xml

# The directory that contains the pepXML and protXML files
srcDir=/net/nunn/vol1/mmudge/2023_02_13_HAB_methodstesting_k.vs.s/MZXMLS_2ug

# The name of the file where results will be saved to
outputFile=/net/nunn/vol1/mmudge/2023_02_13_HAB_methodstesting_k.vs.s/MZXMLS_2ug/ABACUS_output.tsv

# The path the the FASTA formatted file used for the original protein search
# Relative paths are allowed
fasta= /net/nunn/vol1/mmudge/2022_HAB_metagenome/nunn_done/megahit3/HAB.megahit2.proteins.fasta

# The minimum PeptideProphet score the best peptide match of a protein must have
maxIniProbTH=0.99

# The minimum PeptideProphet score a peptide must have in order to be even considered by Abacus
iniProbTH=0.50

# E.P.I: Experimental Peptide-probability Inclusion threshold
# If a protein does not contain at least one peptide exceeding this PeptideProphet score, none of the
# peptide evidence for this protein will be considered. This is applied on an experiment by experiment case.
epiTH=0

# The minimum ProteinProphet score a protein group must have in the COMBINED file
minCombinedFilePw=0.93
```

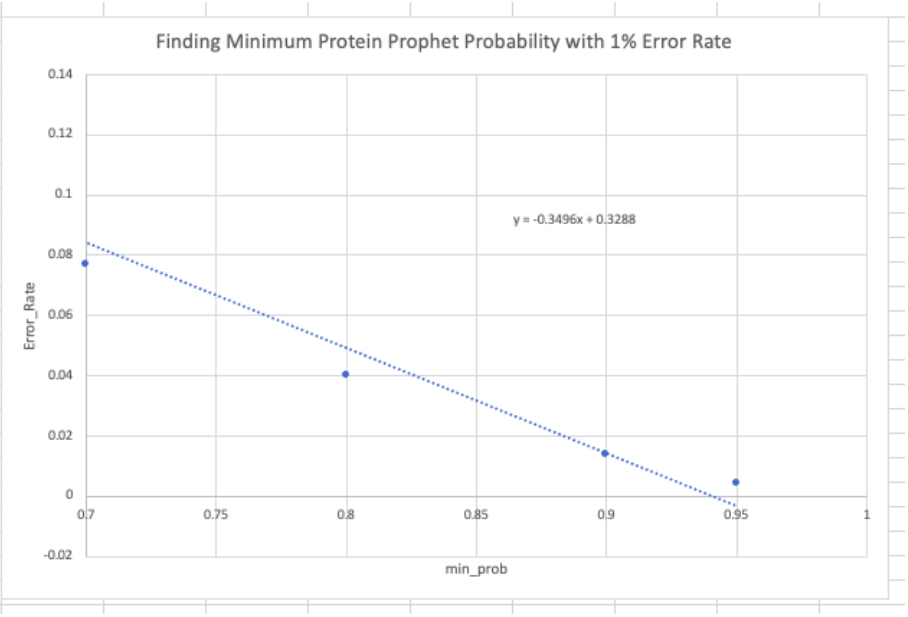
Redo with all 8 samples, new database including enolase

Directory: /net/nunn/vol1/mmudge/2023_02_13_HAB_methodstesting_k.vs.s/MZXMLS_eno

Comet.params Database: /net/nunn/vol1/databases/contam_QC_eno_Apo.fasta

min_prob	Error_Rate
0.95	0.004
0.9	0.014
0.8	0.04
0.7	0.077
0.6	0.128

Lin. Regression	
y	0.01
slope	-0.3496
y-int	0.3288
x	0.9118993




```
#
# ABACUS parameter file
# Generated on: 2016Jun30_1144
#

# Name to give the database
dbName=ABACUSDB

# Name of protXML file corresponding to merged/combined results
combinedFile=/net/nunn/vol1/mmudge/2023_02_13_HAB_methodstesting_k.vs.s/MZXMLS_eno/interact-COMBINED.prot.xml

# The directory that contains the pepXML and protXML files
srcDir=/net/nunn/vol1/mmudge/2023_02_13_HAB_methodstesting_k.vs.s/MZXMLS_eno

# The name of the file where results will be saved to
outputFile=/net/nunn/vol1/mmudge/2023_02_13_HAB_methodstesting_k.vs.s/MZXMLS_eno/ABACUS_output.tsv

# The path the the FASTA formatted file used for the original protein search
# Relative paths are allowed
fasta=/net/nunn/vol1/databases/contam_QC_eno_Apo.fasta

# The minimum PeptideProphet score the best peptide match of a protein must have
maxIniProbTH=0.99

# The minimum PeptideProphet score a peptide must have in order to be even considered by Abacus
iniProbTH=0.50

# E.P.I: Experimental Peptide-probability Inclusion threshold
# If a protein does not contain at least one peptide exceeding this PeptideProphet score, none of the
# peptide evidence for this protein will be considered. This is applied on an experiment by experiment case.
epiTH=0

# The minimum ProteinProphet score a protein group must have in the COMBINED file
minCombinedFilePw=0.91
```

Protein Counts

.prot.xml files: sorting by probability min = 0.9

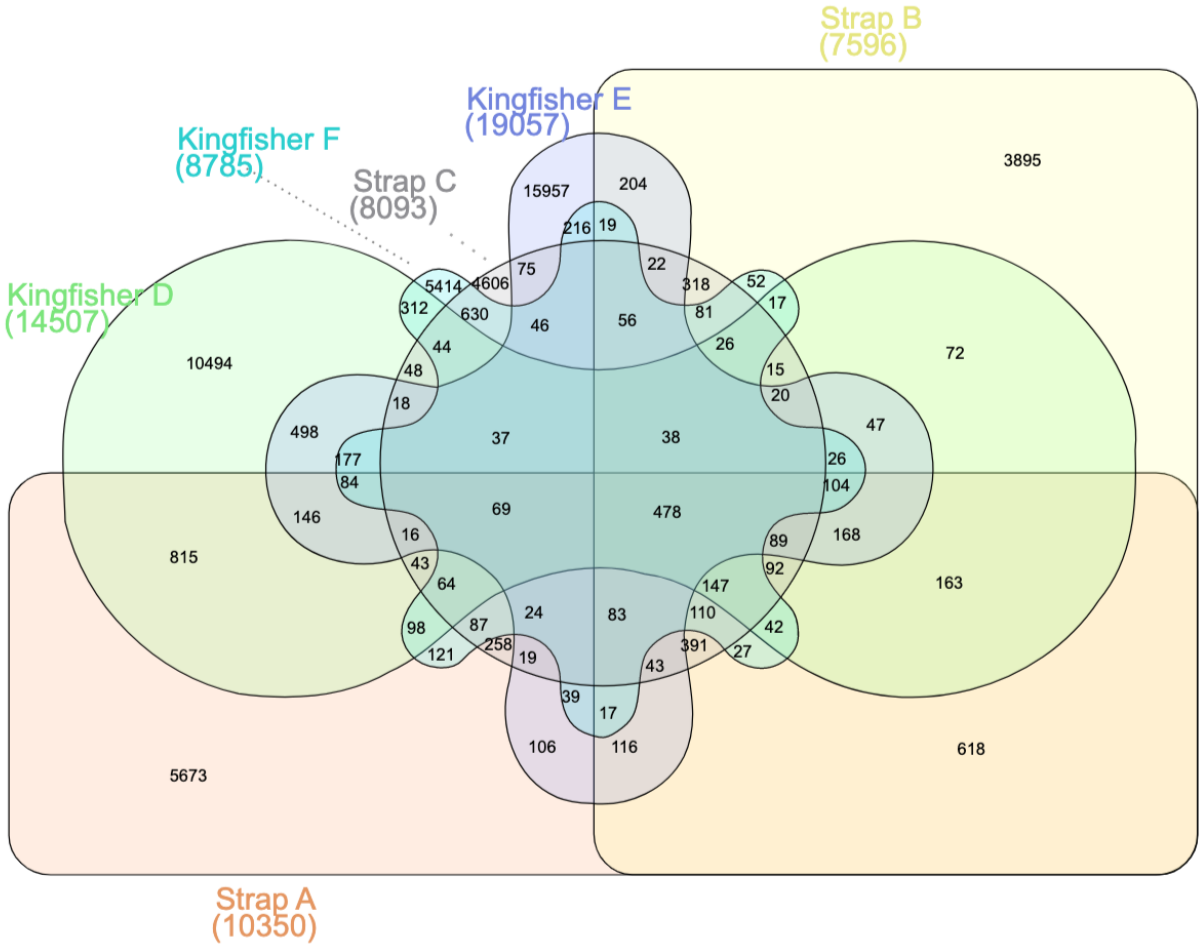
.pep.xml files: summary counts

Sample ID	Day	Time point	Volume Filtered	Num. Proteins prot.xml	Num. Unique Peptides pep.xml	Num. Unique Proteins pep.xml	Num correct Proteins @ Error rate = 0.1 prot.xml	Num. unique enolase peptides
strap A	03	13	2000	2705	**10365**	**6135**	2187	**41**
strap B	07	17	1000	1318	**7604**	**4536**	1224	**27**
strap C	15	17	1000	1420	**8101**	**4753**	1209	**36**
kingfisher D	03	13	2000	2235	**14719**	**8085**	2236	**23**
kingfisher E	07	17	1000	2200	**19273**	**10361**	3186	**37**
kingfisher F	15	17	1000	1624	**9014**	**5185**	1442	**42**
kingfisher D 2 ug					**7884**	**4595**	1394	**31**
kingfisher E 2 ug					**14904**	**7912**	2092	

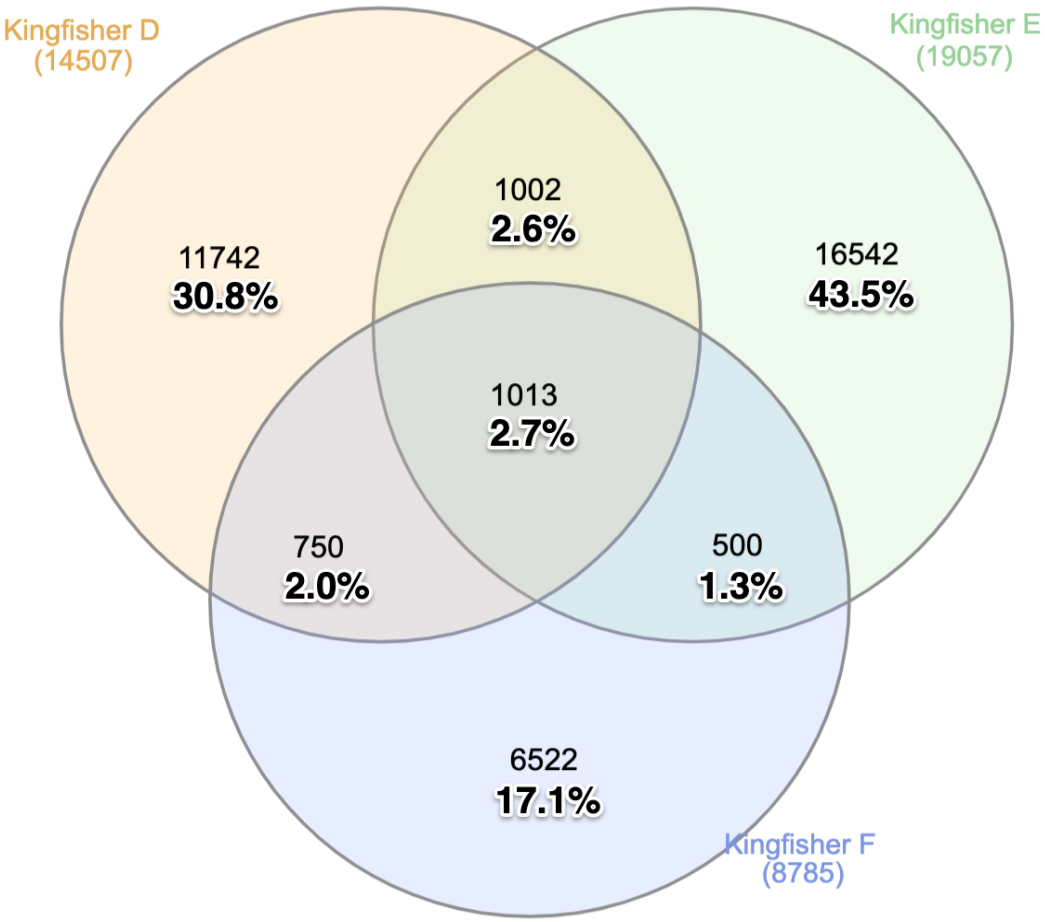
HAB Methods test peptides.xlsx 1 MB

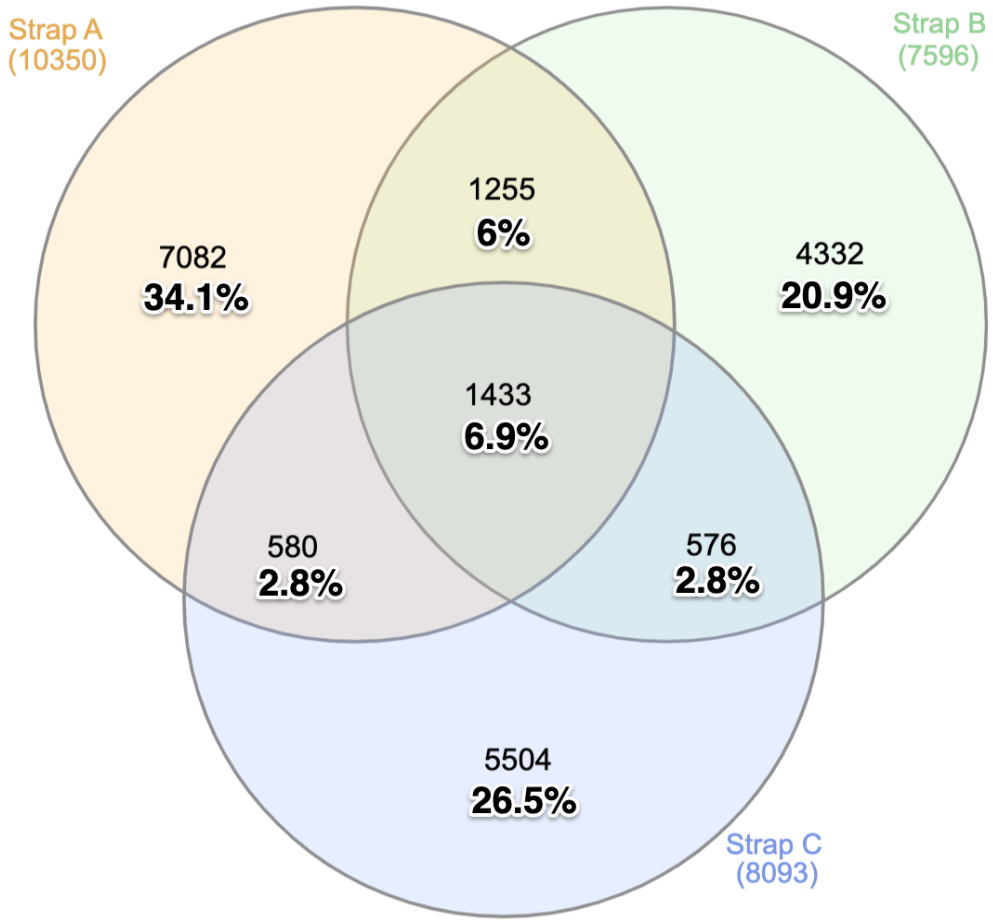
Venn Diagrams

All 6 samples

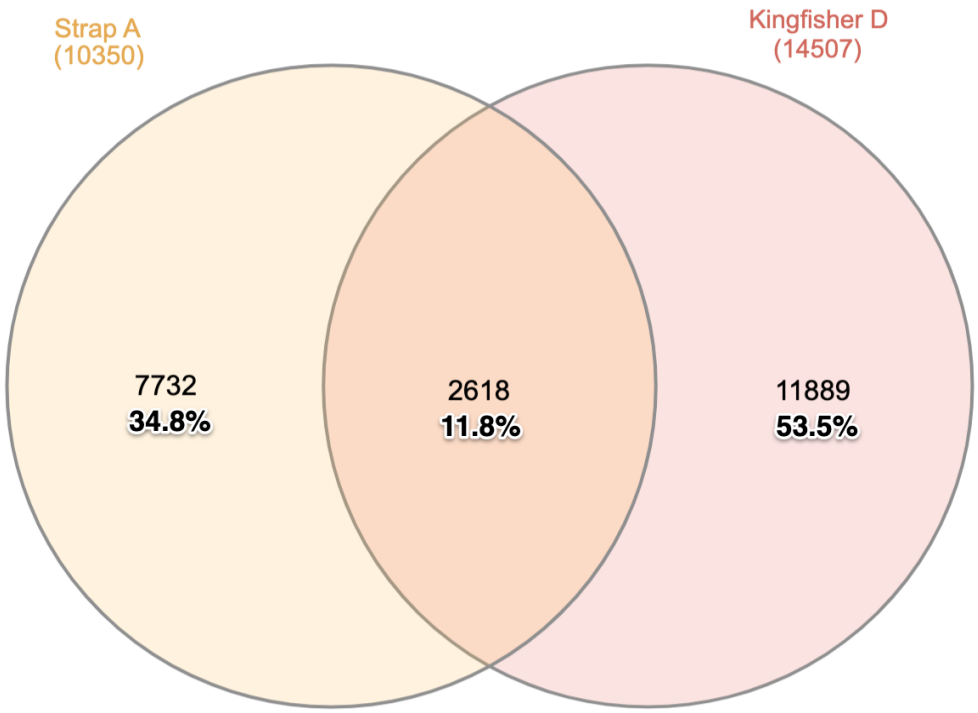


Comparison within methods

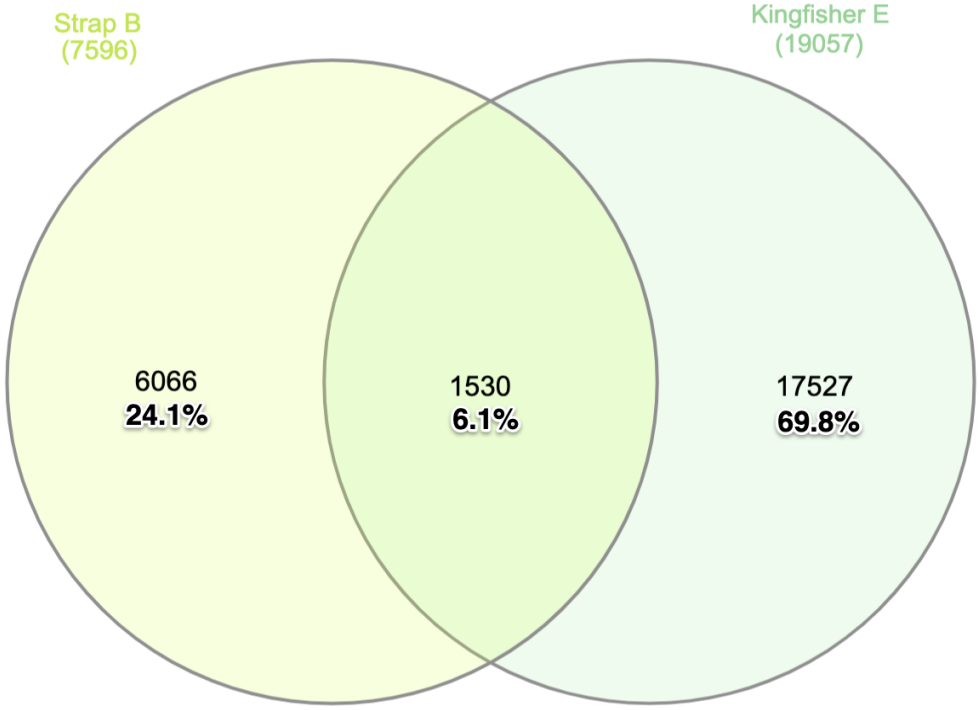

Kingfisher D
(14507)
Kingfisher E
(19057)
Kingfisher F
(8785)
11742
30.8%
1002
2.6%
16542
43.5%
1013
2.7%
750
2.0%
500
1.3%
6522
17.1%



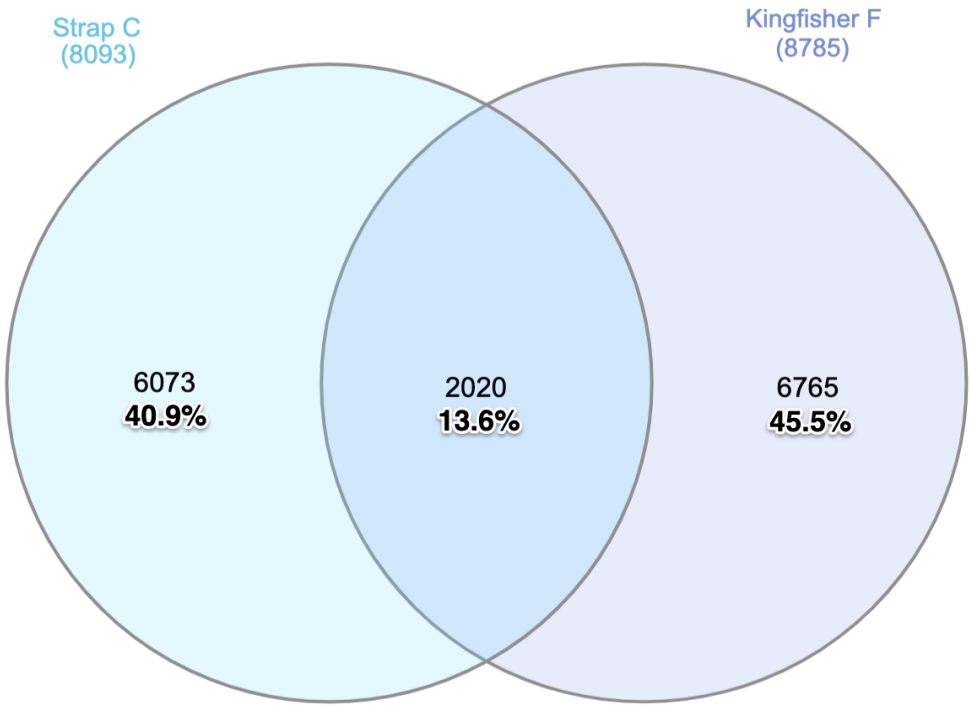
Comparison within timepoints
Day 3 13:00



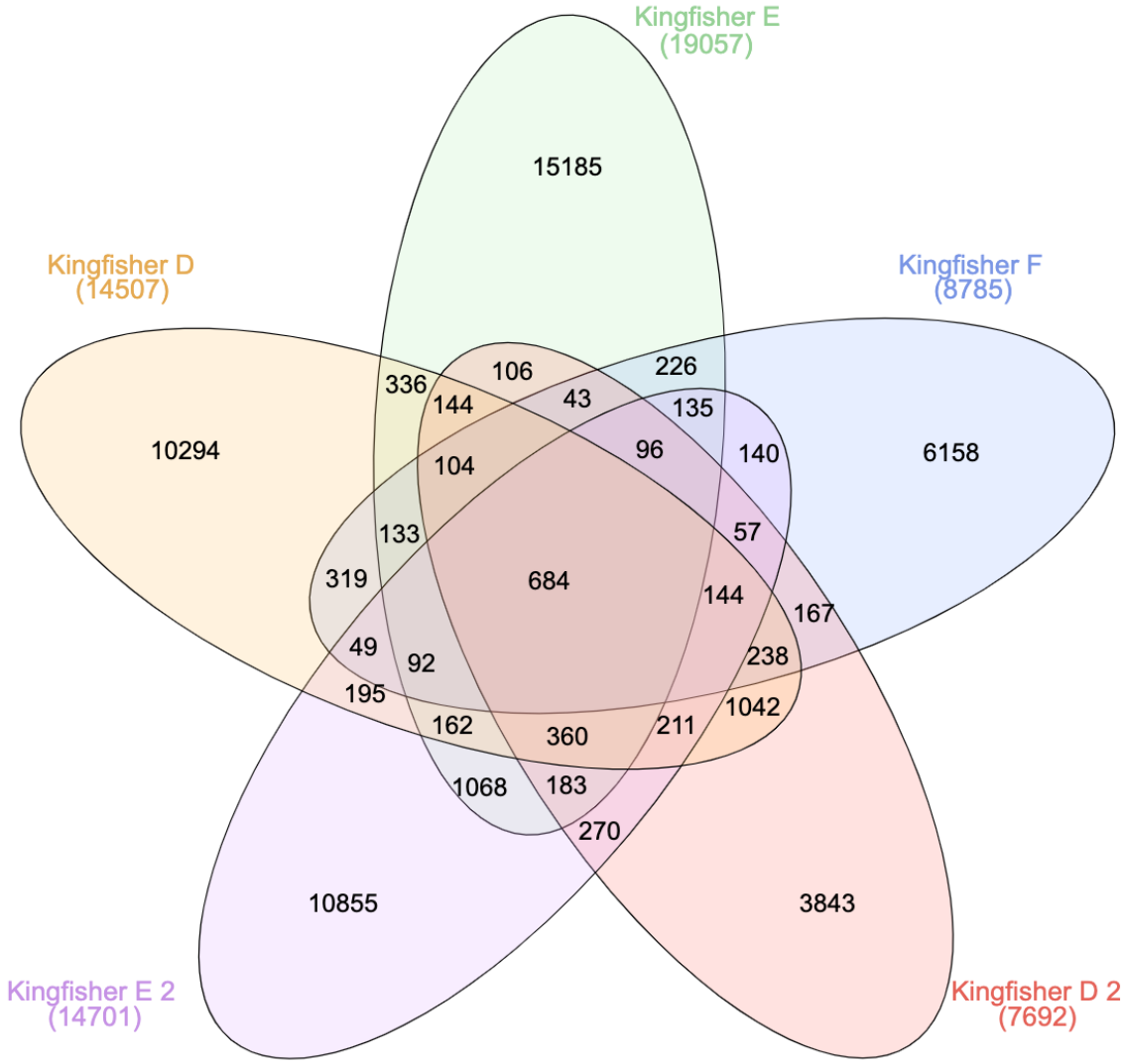
Day 7 17:00



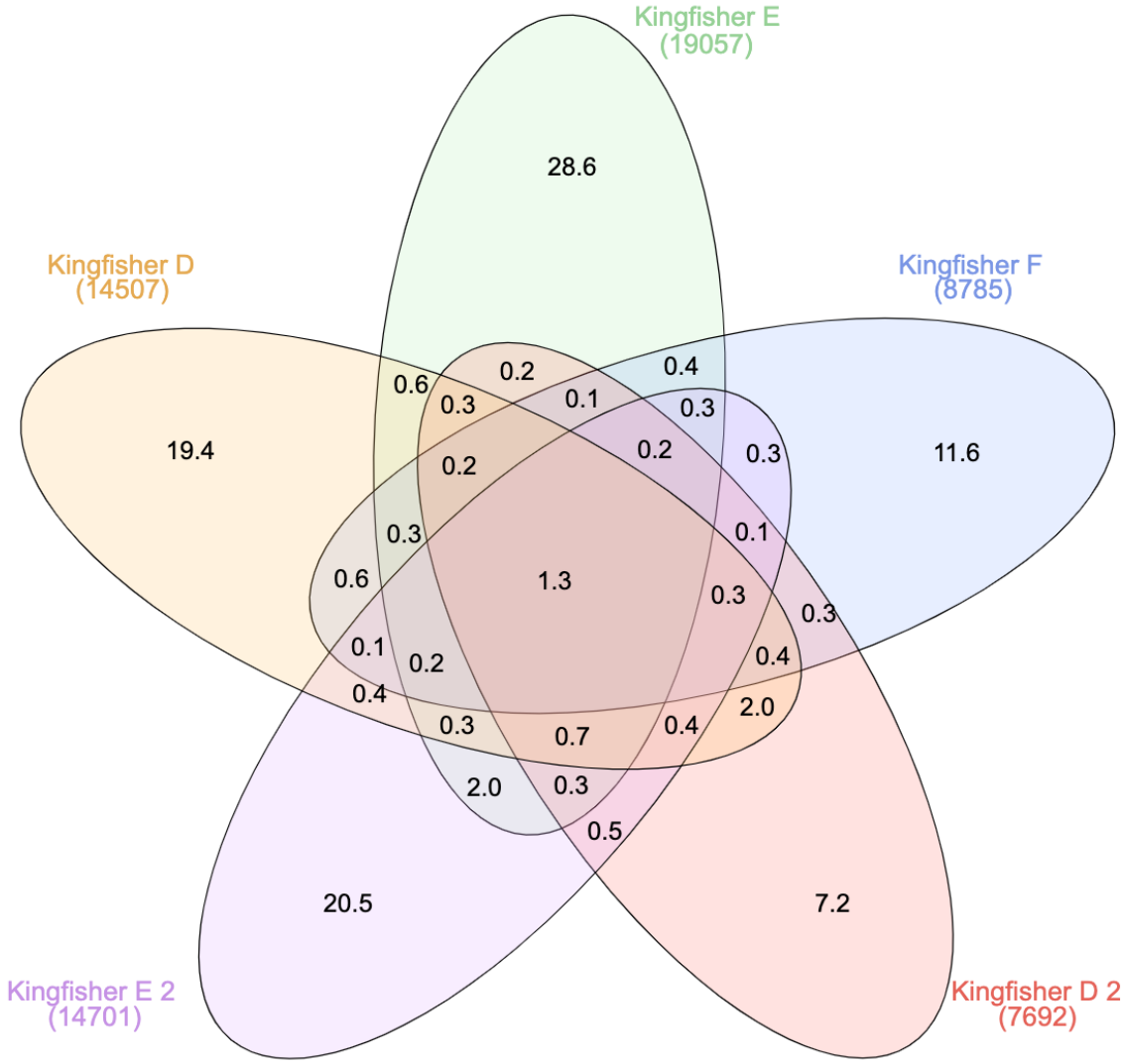
Day 15 17:00



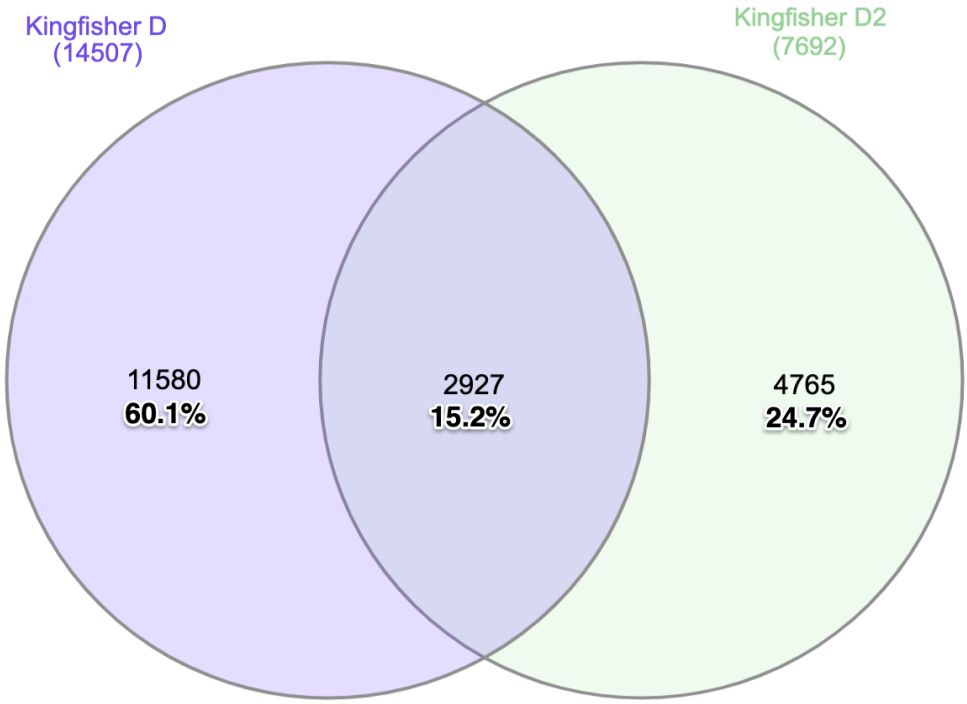
Comparison of 5 Kingfisher runs



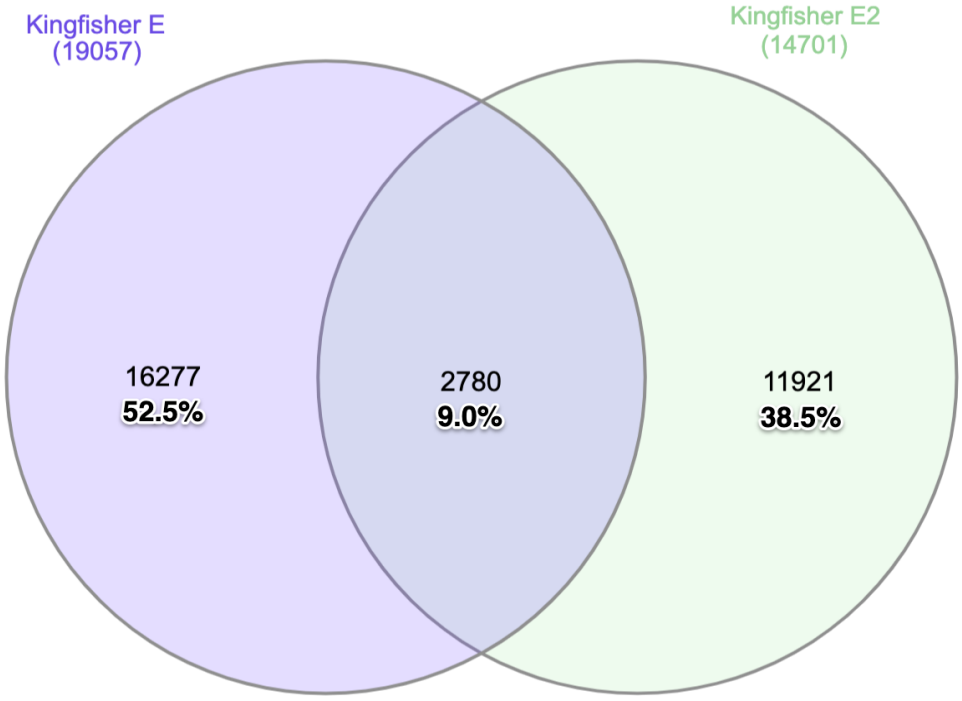
Percentages:



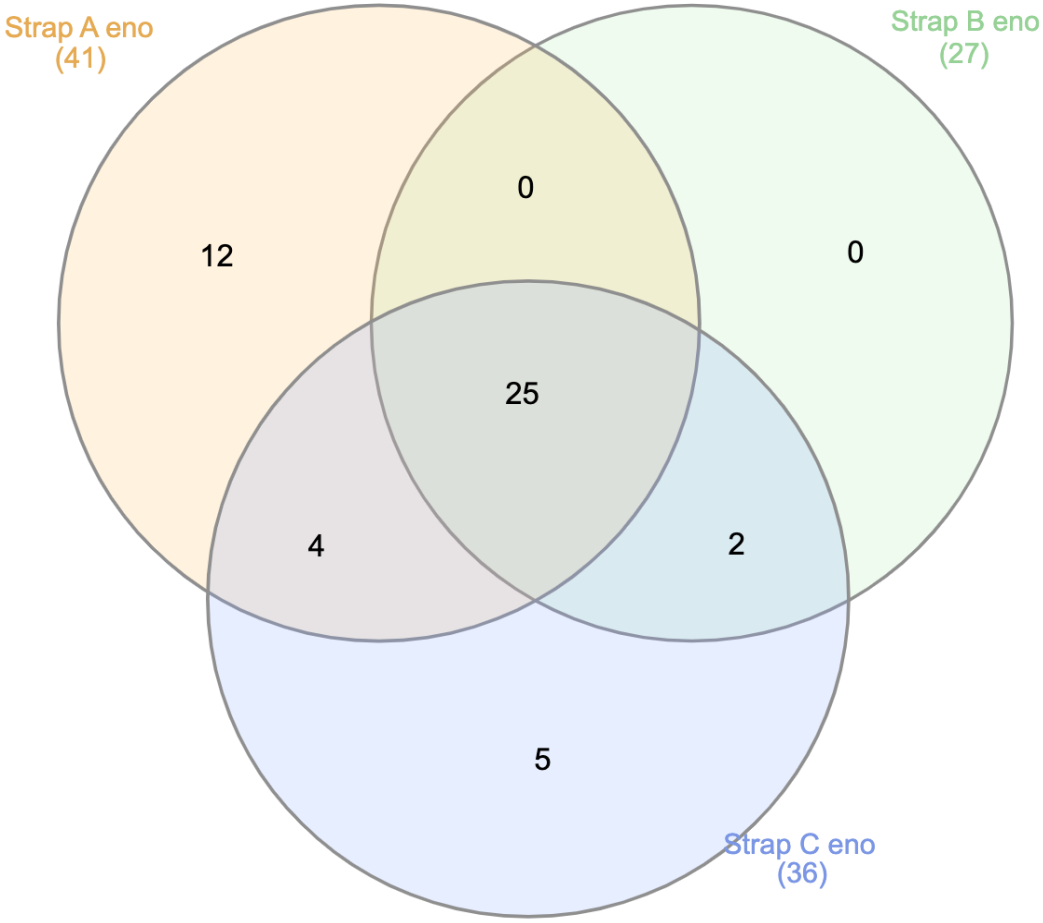
Kingfisher D vs Kingfisher D 2ug

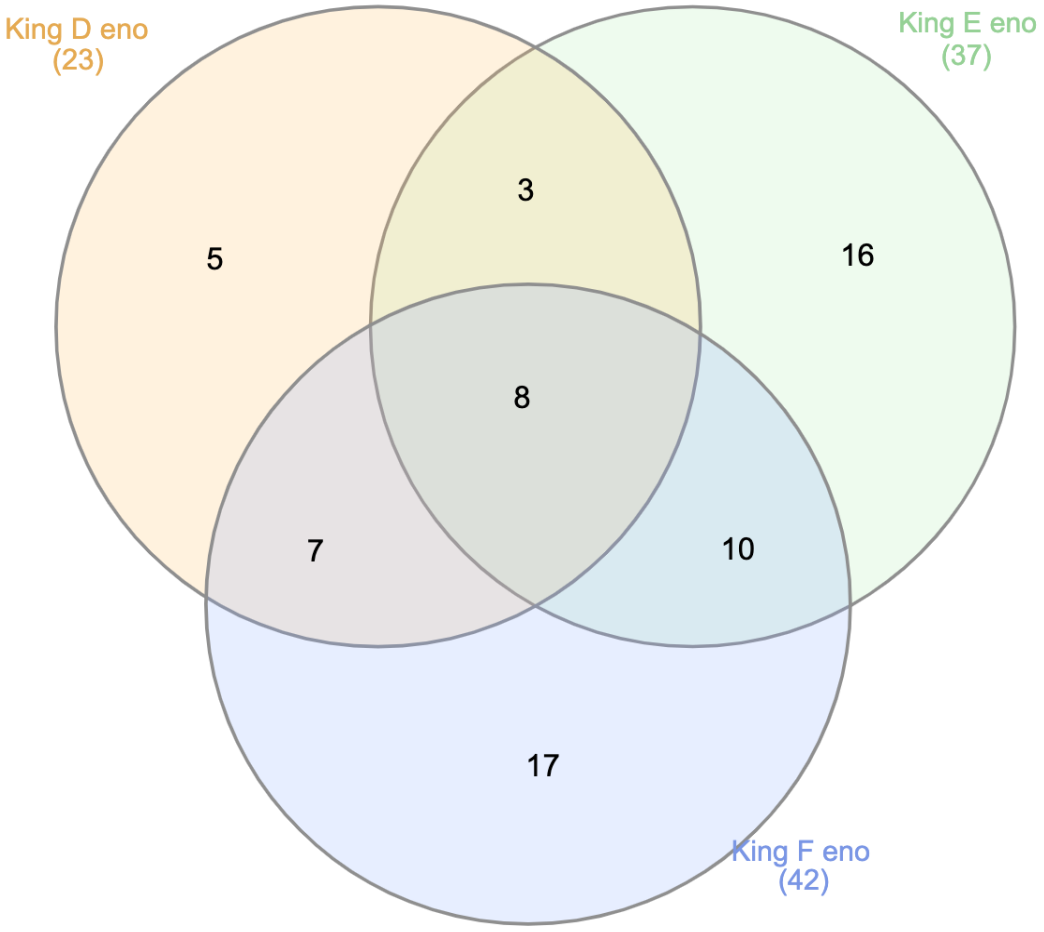


Kingfisher E vs Kingfisher E 2ug



With Enolase


Strap A eno
(41)
Strap B eno
(27)
Strap C eno
(36)
12
0
0
25
4
2
5



HAB Methods Testing Mass Spec

Sample Name	Digestion Method	Vial Location	
		PRTC	no PRTC
strap_A	S-trap	B6	C3
strap_B		B3	C5

strap_C	B5	C2	
kingfisher_D	Kingfisher	B1	C6
kingfisher_E		B4	C4
kingfisher_F		B2	C1

Sample Prep:

1. Diluted stock PRTC to 500 fmol/uL:
    - 4 uL stock 5 pmol/uL PRTC
    - 36 uL 0.1% Formic acid
2. Made samples
    - 10 ul 0.5 ug/ul sample
    - 1.0 ul 250 fmol/ul PRTC
    - 4.0 ul 0.1% formic acid

Attaching Columns

1. Connected trap with frit (want 2-3 mm) pointed away from column input line (~4 cm)
2. Maintenance > Prepare > Isocratic Flow > Parameters
    - Volume [uL]: 100.00
    - Flow [uL/min]: 0.2
    - AB Mix [%B]: 100
3. Ran for 2-3 min, let pressure hold around 200
4. Increased flow to 0.5 uL/min, hold for another 2-3 min
5. Attached Analytical column
    - Volume [uL]: 100.00
    - Flow [uL/min]: 0.2
    - AB Mix [%B]: 6
6. Ran for 10 min, let pressure hold around 200
7. Increased gradient to 75% B, run 10 min
8. Increased flow to 0.3 uL/min, run 10 min
9. Decreased gradient to 6% B, run 10 min, plus approx. 15 min due to red sharpie ink going through after wiping end with methanol

NOTE: Trap kept leaking, had to remove and recut ends/reattach multiple times. Could be due to back pressure from analytical column, eventually did stop leaking.

After Running

1. Maintenance > Prepare > Isocratic Flow > Parameters
    - Volume [uL]: 100.00
    - Flow [uL/min]: 0.3
    - AB Mix [%B]: 75
2. Ran for ~5 min
3. Decreased gradient to 6% B, ran ~10 min

HAB Methods Testing 3 (SDS vs TEAB)

QUESTION: How comparable are the microbiomes extracted to the 2021 6-day period?

12 total samples
Comparing SDS squish method vs TEAB incubation & s-trap vs Kingfisher for both

Filter Info					Filter Processing Method	Tubes				BCA Results		Digestion Method	Notes
Sample Name	Volume Filtered	Day	Timepoint	Filter letter		empty weight (g)	with sample weight (g)	water to add (uL)*	Speed vac time	Final Concentration (ug/uL)	Protein lysate vol. needed for 50 ug		
A	2000	03	13	B	SDS Sq-								

uish	0.991	1.102	-	1h 50m	1.118	44.72	S-trap						
	B	1000	07	17	C	0.992		1.093	-	3h 10m	2.02	24.74	less foa my squish
	C	1000	15	17	E	0.992		1.088	4	3h 10m	1.185	42.21	less foa my squish
	D	2000	03	13	C	0.997	1.025	72	1h 50m	0.80	62.40	Kingfisher	
	E	1000	07	17	B	0.991	1.075	16	3h	1.531	32.65		less foa my squish
	F	1000	15	17	D	0.991	1.054	37	1h 50m	0.55	90.14		less foa my squish

G	2000	03	13	extra (more complete)	TEAB Incubation (with speed vac)	0.986			3h 30m	1.488	33.60	S-trap	
H	1000	07	17	B extra		0.991			3h 30m	1.68	29.74		
I	1000	15	17	A		0.991			3h 40m	1.971	25.36		
J	2000	03	13	extra		0.974			3h 10m	1.85	27.01	Kingfisher	
K	1000	07	17	A		0.989			3h 30m	2.058	24.30		
L	1000	15	17	C	TEAB Incubatio	0.990			3h 40m	2.41	20.76		
M	1100	15	13	D									

suspending pellet)				n/a	0.181	276.51	S-trap						
	N	1100	15	13	B					n/a	0.20	252.01	
	O	1000	07	21	B extra					n/a	0.273	182.91	
	P	1000	07	21	B				n/a	0.32	158.66	Kingfisher	
	Q	1750	03	09	B				n/a	0.244	204.65		
	R	1750	03	09	C				n/a	0.19	264.78		

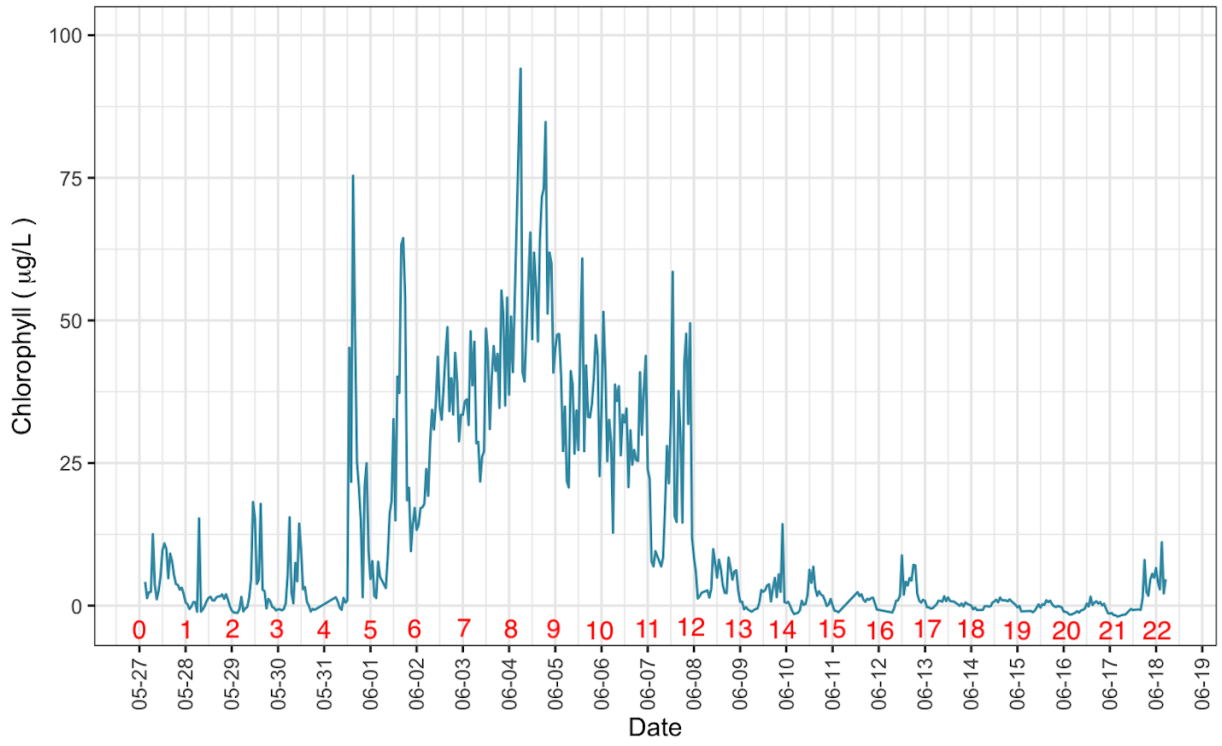
*[(empty + 0.1) - w/ sample]

2/2/23 - 2/3/23

Cataloging and Selecting Filters

- Go through a box at a time on dry ice and re-bag filters in uncracked ziplock bags
  - Take pictures of labels on old bag with volumes filtered
- Select 3 timespoints to take 4 filters from each for methods testing: 5/30 1 pm, 6/3 5 pm, 6/11 5 pm
  - leave the highest filtered vol
- Select 5 timepoints to take 3 filters from each for triplicates: 6/4 9 am, 6/7 1 am, 6/2 5 pm, 5/29 1 am, 6/15 9 pm (2 filters)
- Select 1 filter each from the remaining timepoints
  - Use those with highest volumes filtered

🔗 2021 HAB FIlter Masterlist.xlsx



2/6/23

SDS squish

Used highest vol filters (leave max vol)

SDS Buffer:

For 1,000 uL:

- 50 uL 1M TEAB (cold room)
- 250 uL 20% SDS (bench)
- 2 uL 1 M MgCl2 (bench)
- 688 uL HPLC water (above bench)
- 10 uL 100X HALT protease & phosphatase inhibitors (cold room, come in 100uL tubes)

1. Label and weigh tubes
2. Add 100 uL SDS buffer on top of filter in bag
3. Squish 1 min
4. Transfer liquid to eppie tube
5. Add 100 uL HPLC water on top of filter in bag

6. Squish 1 min
7. Repeat step 5-6 2x for a total of 3 washes
8. Transfer liquid to eppie tube

NOTE: C and F squish were way less foamy than other squishes

TEAB Incubation

TEAB wash:

For 6 mL:

- 60 uL 100X HALT protease & phosphatase inhibitors
- 5,940 uL 1M TEAB

Store on ice

1. Add 1 mL prot inhibitors + TEAB solution on top of filter in bag
2. Incubate filter completely covered in ice for 10 min
3. Squeeze liquid to bottom of bag and pipette into eppie tube

Sonication

1. Sonicate samples w/ probe
    1. Speed 3 for 10 sec then placed on ice for 30 sec
    2. 5 times total for each sample
2. Spin in large centrifuge @ 4°C and 10,000g for 10 min to pellet the cell debris (TEAB)
3. Pipette supernatant to new tube (TEAB)

Speed Vacuum

- SDS squish: ~400 uL -> approx. 2 hours
    - Speed vac to 100 uL
- TEAB Incubation: ~1 mL -> approx. 4 hours
    - speed vac to dry

2/7/23

BCA Assay

Resuspend TEAB samples in 100 uL SDS buffer

Only do one well per sample!

- Compare final concentrations & volume needed to got 50 ug protein
    - Which has higher concentrations?
    - Which is <40 uL?

1. CALCULATE 50:1 ratio A:B
    1. [12 samples + (9 standards + 1 blank) * 3] * 200 uL = Volume Reagent A
        1. 42 * 200 uL = 8,400 uL -> 8,500 uL
    2. Volume Reagent A / 50 = Volume Reagent B
        1. 8,500 uL / 50 = 170 uL
2. Add Reagent A + Reagent B to a falcon tube and vortex (BCA box above bench)
3. Add 10 uL standards (A-I) and 10 uL blanks (SDS buffer) along top 3 rows of the plate with replicates in columns
4. Add 10 uL samples along next row
5. Add 200 uL Reagent solution to every occupied well (do standards last)
6. Read plate using Varioskan
    1. Incubate plate @ 37°C for 30 min
7. Fill out BCA Excel sheet to find sample protein concentrations in ug/mL and uL volume needed for 50 ug of protein
8. Freeze in -80

TEAB samples were very white, a little goopy. Protein concentration for redo of TEAB was very low, decided to just digest SDS squish samples.

2/9/23

Digest Proteins

- S-trap 6 samples: 3 SDS
    - Miranda
    - S-trap Protocol
- Process 6 samples on Kingfisher: 3 SDS
    - Rachel
    - Kingfisher Protocol

Kingfisher protocol with notes.pdf 2 MB

Speed Vacuum

- S-trap: ~150 uL -> approx. 1 hour
  - speed vac to dry
- Kingfisher: ~150 uL -> approx. 1 hours
  - speed vac to dry

2/10/23

Pack column

1. Make packing solution
   - 1 mL Acetone
   - 1 scoop C beads
   - wash vial + stir bar with Acetone first!
2. Take out bottom screw of pressure chamber and remove lid
3. Shake packing solution and place in middle
4. Stir @ 350 rpm
5. Place column in metal holder, wipe tip then score and break off bottom end
6. Screw metal holder back in place, hand tighten then screw
   - Make sure column goes in vial
7. Gently guide column down until you feel bottom then bring up slightly
8. Hand tighten washer around column then with wrench 1/2 turn
9. Turn on gas (right) then adjust pressure (left) to approx. 1000 psi
10. Turn dial left of column, flip back lever and you will hear gas
11. Check for solvent at top of column

NOTE: Had to use a thicker packing solution made with methanol to successfully pack

---

2/13/23 - 2/14/23

Mass Spectrometer

- Have from 2/13 @ 10 am to 2/14 @ 6 pm
- 5 QCs
- 6 samples- running each with and without PRTC
- Emma will help set up on Monday 2/13

HAB Stats meeting with Noble group

Rowan
Casanova found peptides and she compared these to the peptides in the meetagenome and peptides IDed from the comet based search DB Miranda generated.
R could use Casanova results to help determine which metagenomic assembler is more accurate

Rich discovered that Casanova does something

M- Rowan would like RAIN analysis results from DDA data

Mike is writing code to pull out a level to report
opt 1 delet all notes less than threshold T and keep the leaves above
opt 2 delete all nodes with no siblings >T and keep the leaves.

Brook proposed we generte a control (T0) and use metagomics to test each later timepoint against that
Issue of FDR control on the p-values

Miranda wants to cluster GO terms through time.

HAB Methods Testing 2 1/30/23

3 total samples
Started at 1:15, last sample put in -80 at 4:10
Time stamp | Volume | Reagent | Timing/Temperature/Speed | Notes

W HAB Methods Testing Results 0131... 16 kB

Sample Name	Volume filtered (mL)	Method	Protein concentration (ug/uL)
C	700	TEAB incubation + probe	1.594
I	700	TEAB incubation + probe	1.390
D	600	Bead beating	0.643

Make TEAB wash

For 2 mL:

- 20 uL 100X HALT protease & phosphatase inhibitors
- 1,980 uL 1M TEAB

Store on ice

Make SDS buffer without SDS

For 750 uL:

- 50 uL 1M TEAB (cold room)
- 2 uL 1 M MgCl2 (bench)
- 688 uL HPLC water (above bench)
- 10 uL 100X HALT protease & phosphatase inhibitors (cold room, come in 100uL tubes)

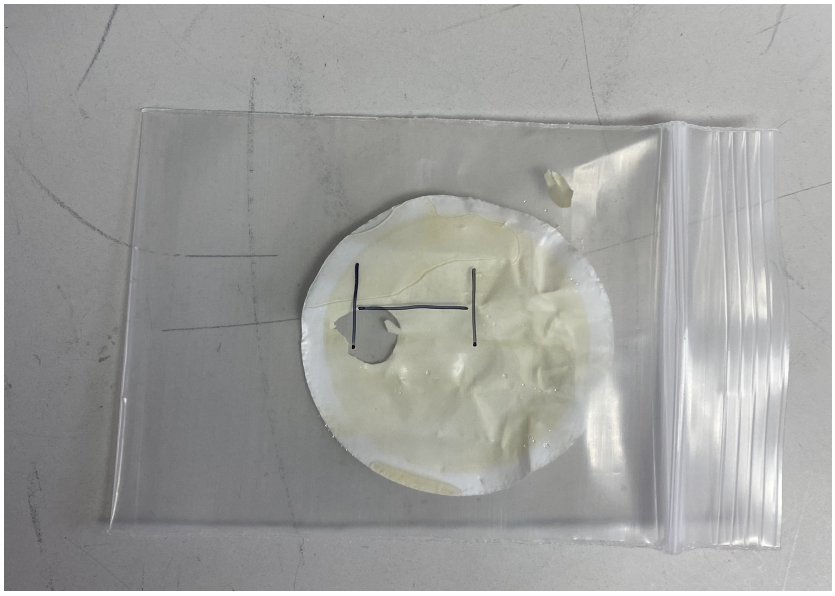
TEAB Incubation + Probe Sonication

Rerun probe sonication using TEAB incubation instead of squishing
Number of samples: 2

1. Added 1 mL prot inhibitors + TEAB solution on top of filter in bag 1:40
2. Incubated filter completely covered in ice for 10 min

3. Squeezed liquid to bottom of bag and pipetted into eppie tube
4. Sonicated samples w/ probe 2:00
    1. Speed 3 for 10 sec then placed on ice for 30 sec
    2. 5 times total for each sample
5. Spun in large centrifuge @ 4°C and 10,000g for 10 min to pellet the cell debris 2:17
6. Pipetted supernatant to new tube
7. Froze in -80

NOTES: Filter had hole: tried to get all pieces into bag



Bead Beating (w/o SDS)

Rerun bead beating with 500 uL buffer w/o SDS (increased from 300 uL) and no addition of SDS after centrifuge, after speed vac resuspending in SDS buffer
Number of samples: 1

1. Added 100 uL prot inhibitors + TEAB solution on top of filter in bag 2:15
2. Incubated filter in ice for 10 min
3. Cut filter into small squares using razor blades on petri dish on ice 2:47
4. Placed squares in twist cap tube
5. Added 0.25 mL 0.5 mm Yttria Stabilized Zirconium Oxide beads
6. Added 500 uL buffer w/o SDS
7. Bead beat for 1 min then let sit 1 min 4 times 3:10

    1. Took block out and put on ice for 1 min after 2nd shaking
8. Poked 3 holes in bottom of tube with heated pin 3:40
9. Ethanol wiped bottom of tube
10. Placed tube in rounded 2 mL eppie tube
11. Centrifuged at 3,000 RPM for 1 min - closer to a few sec, 500 uL of liquid was able to go through
12. Pipetted out liquid from 2 mL eppie to new tube, avoiding any beads that made it through
13. Spun in large centrifuge @ 4°C and 10,000g for 10 min to pellet the cell debris 3:55
14. Pipetted supernatant to new tube
15. Froze in -80

NOTES: If bead beating is the method we pursue in future, will want to autoclave supplies including the beads. While the twist top tubes can be shoved into the 2 mL eppie tubes (with force) they are TOO TALL for mini centrifuge, had to stop immediately but 500 uL had gone through in those couple seconds.

1/31/23

Made S-trap Solubilization/Lysis Buffer (SDS Buffer)

For 1,000 uL:

- 50 uL 1M TEAB (cold room)
- 250 uL 20% SDS (bench)
- 2 uL 1 M MgCl2 (bench)
- 688 uL HPLC water (above bench)
- 10 uL 100X HALT protease & phosphatase inhibitors (cold room, come in 100uL tubes)

1. Speed vacuumed to dry 10:26
2. Resuspended pellet in 100 uL SDS buffer 12:55 (D), 2:30 (I), 2:45 (C)

NOTES: No samples were goopy! Bead beating sample was more viscous but still easily pipett-able. Sonication samples were more green tinted.

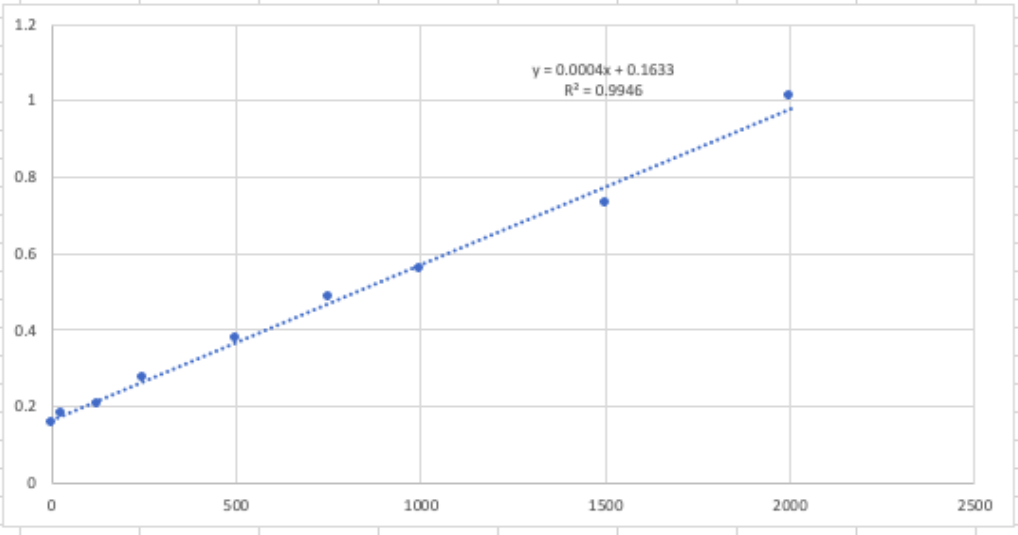
BCA Assay

1. CALCULATE 50:1 ratio A:B

    1. [(3 samples + 9 standards + 1 blank) * 3] * 200 uL = Volume Reagent A
        1. 39 * 200 uL = 7,800 uL -> 8,000 uL
    2. Volume Reagent A / 50 = Volume Reagent B
        1. 8,000 uL / 50 = 160 uL
2. Added Reagent A + Reagent B to a falcon tube and vortex (BCA box above bench)
3. Added 10 uL standards (A-I) and 10 uL blanks (SDS buffer) along top 3 rows of the plate with replicates in columns
4. Added 10 uL samples along next 3 rows of the plate with replicates in columns
5. Added 200 uL Reagent solution to every occupied well (do standards last)
6. Read plate using Varioskan 3:45
    1. Incubated plate @ 37°C for 30 min
7. Filled out BCA Excel sheet to find sample protein concentrations in ug/mL and uL volume needed for 50 ug of protein
8. Froze in -80

BCA_013123_HAB.xlsx 23 kB

Concentration	Std1	Std2	Std3	Average
0	0.15268	0.154981	0.163218	0.1569597
25	0.173808	0.182589	0.179994	0.178797
125	0.204544	0.206716	0.209402	0.2068873
250	0.287308	0.268291	0.263347	0.272982
500	0.374336	0.373493	0.387592	0.3784737
750	0.517464	0.475737	0.466975	0.4867253
1000	0.564595	0.555637	0.557645	0.5592923
1500	0.751705	0.727314	0.716271	0.7317633
2000	1.03789	0.996685	0.994542	1.0097057



Sample Name	rep1	rep1 con. (ug/mL)	rep2	rep2 con. (ug/mL)	rep3	rep3 con. (ug/mL)	Average con. (ug/mL)	converting units- Final Concentration (ug/uL)	Protein lysate vol. needed for 50 ug S-Trap (uL)
C	0.774465	1527.913	0.815672	1630.930	0.812775	1623.688	1594.177	1.594	31.36
I	0.642427	1197.818	0.752569	1473.173	0.763482	1500.455	1390.482	1.390	35.96
D	0.390679	568.448	0.436073	681.933	0.434685	678.463	642.948	0.643	77.77

Y-int	0.1633
slope	0.0004

Mass Spec Protocol

Assembling Column

1. Connect trap with frit (want 2-3 mm) pointed away from column input line
    - Make sure to cut a small piece off the end (make a straight cut) anytime it is pushed through a fitting
2. Under "Maintenance" tab select "Prepare" and "Isocratic Flow"
3. Under Parameters tab input:
    - Volume [uL]: 100.00

    - Flow [uL/min]: 0.2
    - AB Mix [%B]: 100
4. Run for 2-3 min, let pressure plateau and hold around 200
5. Increase flow to 0.5 uL/min, repeat step 4
6. Attach Analytical column, run with
    - Volume [uL]: 100.00
    - Flow [uL/min]: 0.2
    - AB Mix [%B]: 6
7. Wait for a drop to form on end and pressure plateau around 200 then run for approx. 10 min
8. Increase gradient to 75% B, repeat step 7
9. Increase flow to 0.3 uL/min, repeat step 7
10. Decrease gradient to 60% B, repeat step 7

Brook Method:

1. Press "Stop" then under the "Test" menu press "Leaks"
2. Let run, looking for any liquid coming out meaning you have to redo the attachment making it tighter
3. Check pre-column has about 2-3 cm of packing material (lighter color than empty column)
4. Cut off any dead space and run pre-column for 10-15 min

Preparing Samples

1. Make the QC and the blank
    - QC is
    - Blank is 0.1% Formic Acid
2. Add 50 uL 0.1% Formic acid to sample for final concentration 1 ug/uL
3. Vortex samples then centrifuge @ 10,000 for 5 min
4. Dilute stock PRTC to 500 fmol/uL:
    - 4 uL stock 5 pmol/uL PRTC
    - 36 uL 0.1% Formic acid
5. Dilute stock PRTC to 250 fmol/uL:
    - 4 uL stock 5 pmol/uL PRTC
    - 76 uL 0.1% Formic acid
6. Add 1 uL 250 fmol/uL PRTC to autosampler vials, vortexing before pipetting
    - Go all the way to the bottom to avoid bubbles
7. Add 9 uL 0.1% Formic Acid to autosampler vials

8. Add 5 uL 1 ug/uL sample to autosampler vials, keeping at angle to avoid particulates
9. Spin samples in speed vac centrifuge to get rid of bubbles, ~3 min

Starting Run

1. Open Microsoft Connect to get new login and key code (do not refresh!)
2. Take screenshot for Evernote and copy the security code
3. Queue 5 QCs (take ~60 min each plus 17 min to load next one)
4. Look for distinct peaks where preloaded peptides are registering, QCs should look very similar to each other
5. Take a screenshot of each one for Evernote
6. First QC may look a little weird

Cleaning Column (optional)

1. Under "Maintenance" tab select "Prepare" and "Isocratic Flow"
    - Volume [uL]: 100.00
    - Flow [uL/min]: 0.3
    - AB Mix [%B]: 75
2. Let run for 10 min at a steady pressure
3. Decrease %B to 6
4. Let run for 10 min at a steady pressure
5. Stop flow and start take down steps

Taking Down Mass Spectrometer

1. Double check files are moved over
2. Copy over SLD file
3. Export (in Excalibur) sequence as a .csv file
4. Make sure you have all screenshots in Evernote
5. Unplug Voltage
6. Back needle away and carefully remove
    1. Tape in box
7. Unhook horizontal column
8. Hit "stop" button
9. Clean up and return everything to where it goes
10. Home > Eject to remove samples, QC, blank

7 total samples
Started at 10:40, all samples put in -80 at 3:10
Time stamp | Volume | Reagent | Timing/Temperature/Speed | Notes

HAB Methods Testing Results 0127... 19 kB

Sample Name	Volume filtered (mL)	Empty tube weight (g)	Tube weight with dried sample (g)	Water to add (uL) *	Squish + Probe sonication	Bead beating	TEAB incubation + PIXUL	Protein concentration (ug/uL)
B	500	0.985	1.072	13	X			0.769
G	700	0.990	1.113		X			0.844
F	1000	1.001	1.067	34		X		0.181
J	1100	0.984	1.084			X		0.145
A	1000						X (well A1)	0.374
E	950						X (well B1)	0.403
H	700						X (well C1)	0.341

*[(empty + 0.1) - w/ sample]

Squish + Probe Sonication

Number of samples: 2

10:40

Make S-trap Solubilization/Lysis Buffer (SDS Buffer)

100 uL used for squish, 100 uL used for TEAB incubation

200 uL * 5 samples = 1,000 Total Volume Needed

For 1,000 uL:

- 50 uL 1M TEAB (cold room)
- 250 uL 20% SDS (bench)
- 2 uL 1 M MgCl2 (bench)
- 688 uL HPLC water (above bench)
- 10 uL 100X HALT protease & phosphatase inhibitors (cold room, come in 100uL tubes)

Make SDS buffer without SDS

For 750 uL:

- 50 uL 1M TEAB (cold room)
- 2 uL 1 M MgCl2 (bench)
- 688 uL HPLC water (above bench)
- 10 uL 100X HALT protease & phosphatase inhibitors (cold room, come in 100uL tubes)

Make TEAB wash

For 4 mL:

- 40 uL 100X HALT protease & phosphatase inhibitors
- 3,960 uL 1M TEAB

Store on ice

11:10

Squish Method

1. Labeled and weigh tubes
2. Added 100 uL SDS buffer on top of filter in bag
3. Squished 1 min
4. Transfered liquid to eppie tube
5. Added 100 uL HPLC water on top of filter in bag
6. Squished 1 min
7. Repeated step 5-6 2x for a total of 3 washes
8. Transfered liquid to eppie tube

11:45

Probe Sonication

1. Sonicated at speed 3 for 10 sec then place on ice for 30 sec
    1. 5 times total for each sample
    2. Cleaned probe with ethanol in between samples
2. Speed vacuumed down to approx. 100 uL

NOTES: Spun down for 10 min @ 10,000 then transferred supernatant to new tubes

---

Bead Beating

Number of samples: 1

12:05

Bead Beating

1. Cut filter into small squares using razor blades on petri dish on ice
2. Placed in 1.5 mL eppie tube
3. Added 0.25 mL 0.5 mm Yttria Stabilized Zirconium Oxide beads
4. Added 300 uL buffer w/o SDS to tube
5. Bead beat for 1 min then let sit 1 min x 4 times
6. Poked 3 holes in bottom of tube with heated pin
7. Placed tube in rounded 2 mL eppie tube
8. Added 100 uL SDS to tube
9. Centrifuged at 3,000 RPM for 1 min
10. Pipetted out liquid from 2 mL eppie to new tube, avoiding any beads that made it through
11. Discarded 1.5 mL and 2 mL tubes
12. Speed vacuumed down to approx. 100 uL

NOTES: Samples were very goopy after speed vac, spun down and was less cloudy but still unable to pipette. Sample F had 25 min @ 10,000, sample J had 35 min @ 10,000. Attempted to transfer sample F and was unsuccessful, too goopy!

Bead beater instructions:

1. Use frozen metal block and make sure to balance tubes
2. When placing in bead beater ensure that block is above the lip
3. Finger tighten the large black nuts as much as possible
4. Smaller white nuts are to keep black from shaking too much

5. Stand to the side when running and unplug if it starts to rattle

---

TEAB Incubation + PIXUL Sonication

Number of samples: 3

12:50

TEAB Incubation

1. Added 1 mL prot inhibitors + TEAB solution on top of filter in bag
2. Incubated filter in ice for 10 min
3. Pipetted liquid into eppie tube
4. Spun in large centrifuge @ 4°C and 10,000g for 10 min to pellet the cells
5. Discarded supernatant and resuspend cells in 100 uL SDS buffer

2:00

PIXUL Sonication

1. Checked that coupling fluid is approx. 1 inch below reservoir top
2. Loaded samples with 100 uL per well into 96-Well PIXUL Plate, ensuring that every column being sonicated had fluid in all 8 wells
    1. Used water in any wells without sample
3. Sealed plate with adhesive plate seal to prevent samples from spilling
4. Placed in well plate in PIXUL, matching well A1 to top left corner
5. Secured pressure distribution lid and closed external lid
6. Circulated the coupling fluid until it reached approx. 15 C
    1. Waited to start sonication until it reached this temp
7. Set sonication parameters for columns with samples:
    -

Sonication Parameter	DNA** (Cells & Tissue)	Chromatin** (Cells & Tissue)	DNA/RNA** (Purified)	Protein** (Cells & Tissue)
Pulse [N]	50	50	50	50
PRF [kHz]	1.00	1.00	1.00	1.00
Process Time	30:00	30:00	10:00–30:00	1.00–2.00
Burst Rate [Hz]	20.00	20.00	20.00	20

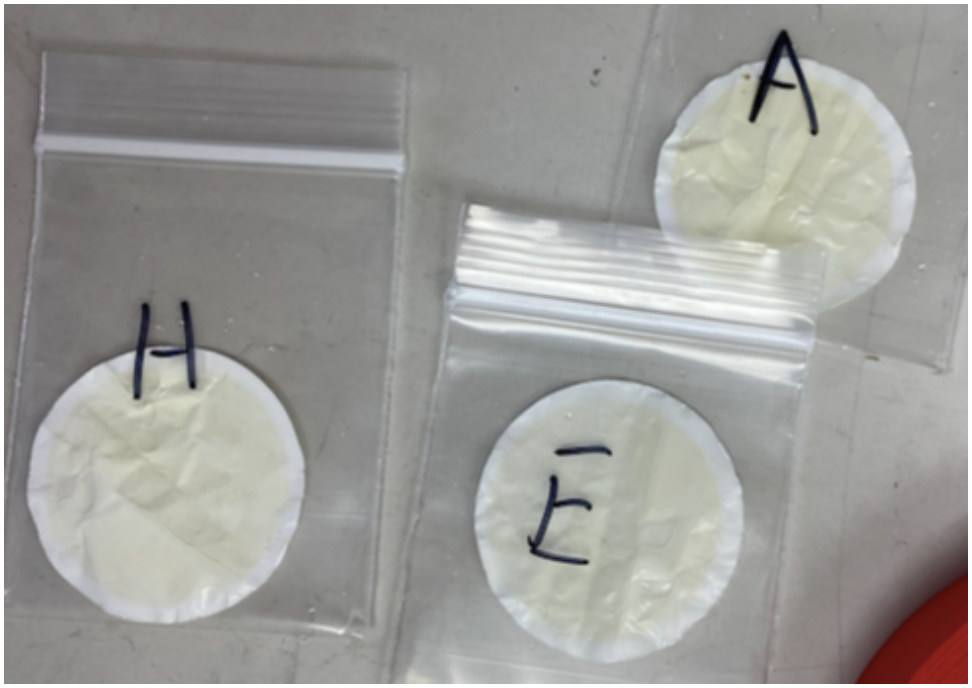
*Labile marks, like phosphorylated epitopes, may be preserved better by discontinuous sonication (e.g. rather than 30 minutes, 4 rounds of 4-6 minutes each, where the PIXUL Coupling Fluid is allowed to circulate and cool between runs).

**Please see our Quick Guides for additional details on sonication recommendations.

8. Started sonication once coupling fluid has reached the appropriate temperature
9. Pipetted out samples into eppie tubes

NOTES: Make sure pressure distribution lid is firmly in pace before starting cooling! Check by trying to lift from long edges. Samples were spun down for 10 min @ 10,000 then the supernatant was transferred to new tubes.

Coupling fluid overflows down front of machine if the pressure distribution lid is not properly locked on



PIXUL operating instructions:

2227.pdf 1 MB

Proteomics Applications with PIXU... 2 MB

Operating the PIXUL Multi-Sample Sonicator

1. Ensure that the PIXUL Coupling Fluid level is about an inch (2.5 cm) below the reservoir top. Do not overfill.
2. Switch on the power switch on the back side of the PIXUL instrument.
3. Press the main power button on the front side of the PIXUL instrument. The touchscreen will start initializing.
4. Load your samples in the 96-well PIXUL plate (cat. No. 53139). 100 µl sample volume per well is optimal. Be sure to keep the outside bottom of the plate clean and free of lint or other debris. Load the sample-containing 96-Well PIXUL Plate (cat. no. 53139) into the PIXUL instrument. Lift the external lid, place the plate with well A1 in the upper left corner, secure down the pressure distribution lid on top of the plate with plate-securing rods, and close the external lid.

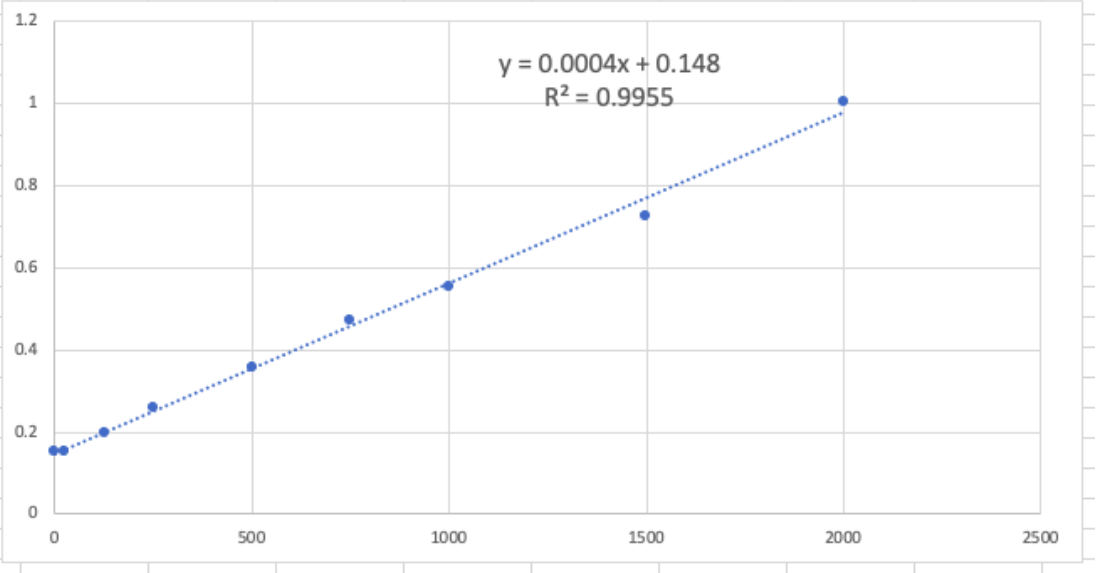
5. On the touchscreen, press Circulate to initiate PIXUL Coupling Fluid cooling. You cannot start the sonication run with out circulation active. You can monitor Coupling Fluid temperature in the upper right hand corner of the touchscreen.
6. On the touchscreen, select the plate columns for which you would like to set sonication parameters. Columns selected together will be outlined in the same color.
7. On the left side of the touchscreen, you can use the left and right arrows to select from saved presets of sonication parameters. You can also use the add and delete buttons to add or remove a row of process settings.
8. Once the PIXUL Coupling Fluid has reached approximately 15°C, press Start on the touchscreen. The time to completion will appear in the top left hand corner of the touchscreen.
9. Once the run has completed, open the external lid. PIXUL Coupling Fluid will drain from underneath the sample plate for the next few seconds.
10. Unload the sample plate by lifting the Pressure Distribution Plate Cover by the Lift Handle and simply pulling up.
11. To turn off the PIXUL instrument, press the main power button on the front side of the PIXUL instrument and switch the power switch off on the back side of the PIXUL instrument.

1/27/23

BCA Assay

1. CALCULATE 50:1 ratio A:B :
    1. [(7 samples + 9 standards + 1 blank) * 3] * 200 uL = Volume Reagent A
        1. 51 * 200 uL = 10,200 uL -> 10,500 uL
    2. Volume Reagent A / 50 = Volume Reagent B
        1. 10,500 uL / 50 = 210 uL
2. Created BCA standards according to manufacturer's instructions if not already made
3. Added Reagent A + Reagent B to a falcon tube and vortex (BCA box above bench)
4. Added 300 uL buffer w/o SDS to sample J, vortexed thoroughly
5. Centrifuged samples J & F @ 10,000g for 10 min and used supernatant for BCA
6. Added 10 uL standards (A-I) and 10 uL blanks (SDS buffer) along top 3 rows of the plate with replicates in columns
7. Added 10 uL samples along next 3 rows of the plate with replicates in columns
8. Added 200 uL Reagent solution to every occupied well (do standards last) and gently shake
9. Read plate using Varioskan
    1. Incubated plate @ 37°C for 30 min
10. Filled out BCA Excel sheet to find sample protein concentrations in ug/mL and uL volume needed for 50 ug of protein
11. Froze in -80

Concentration	Std1	Std2	Std3	Average
0	0.138676	0.17723	0.140057	0.15198767
25	0.157856	0.154296	0.151897	0.154683
125	0.196773	0.197673	0.194985	0.196477
250	0.268461	0.256106	0.258512	0.26102633
500	0.36571	0.351711	0.355233	0.35755133
750	0.490238	0.462443	0.460245	0.47097533
1000	0.572452	0.542631	0.546239	0.553774
1500	0.739104	0.723826	0.718885	0.72727167
2000	1.03477	0.978425	1.0071	1.006765



Sample Name	rep1	rep1 con. (ug/mL)	rep2	rep2 con. (ug/mL)	rep3	rep3 con. (ug/mL)	Average con. (ug/mL)	converting units- Final Concentration (ug/uL)	Protein lysate vol. needed for 50 ug S-Trap (uL)
B	0.429299	703.248	0.479887	829.718	0.457135	772.838	768.601	0.769	65.05
G	0.491354	858.385	0.490151	855.378	0.47478	816.950	843.571	0.844	59.27
F	0.2203	180.750					180.750	0.181	276.63
J	0.204238	140.595	0.208846	152.115	0.204797	141.993	144.901	0.145	345.06
A	0.301956	384.890	0.30593	394.825	0.284395	340.988	373.568	0.374	133.84
E	0.313862	414.655	0.315286	418.215	0.298448	376.120	402.997	0.403	124.07
H	0.285551	343.878	0.28407	340.175	0.283436	338.590	340.881	0.341	146.68

Y-int	0.148
slope	0.0004

A. cervicornis Tephra Search

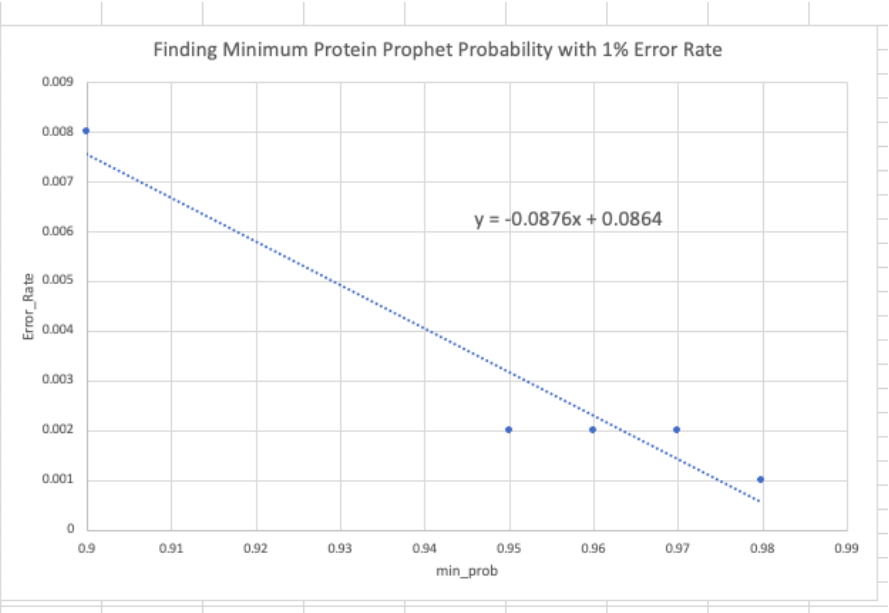
Directory: /net/nunn/vol1/home/rschauer/2021_Dec_10_Cerv
Comet.params Database:
/net/nunn/vol1/databases/Acropora_cervicornis.proteins.symb.contam.fasta

Files:

2021_Dec_10_Maldonado_CORAL_29.mzXML
2021_Dec_10_Maldonado_CORAL_30.mzXML
2021_Dec_10_Maldonado_CORAL_31.mzXML
2021_Dec_10_Maldonado_CORAL_32.mzXML
2021_Dec_10_Maldonado_CORAL_33.mzXML
2021_Dec_10_Maldonado_CORAL_34.mzXML
2021_Dec_10_Maldonado_CORAL_40.mzXML
2021_Dec_10_Maldonado_CORAL_41.mzXML
2021_Dec_10_Maldonado_CORAL_44.mzXML

min_prob	Error_Rate
0.98	0.001
0.97	0.002
0.96	0.002
0.95	0.002
0.9	0.008

Lin. Regression	
y	0.01
slope	-0.0876
y-int	0.0864
x	0.8721461



```
#
# ABACUS parameter file
# Generated on: 2016Jun30_1144
#

# Name to give the database
dbName=ABACUSDB

# Name of protXML file corresponding to merged/combined results
combinedFile=/net/nunn/vol1/home/rschauer/2021_Dec_10_Cerv/interact-COMBIN
ED.prot.xml

# The directory that contains the pepXML and protXML files
srcDir=/net/nunn/vol1/home/rschauer/2021_Dec_10_Cerv/

# The name of the file where results will be saved to
outputFile=outputFile=/net/nunn/vol1/home/rschauer/2021_Dec_10_Cerv/ABACUS
_output.tsv

# The path the the FASTA formatted file used for the original protein sear
ch
# Relative paths are allowed
fasta=/net/nunn/vol1/databases/Acropora_cervicornis.proteins.symb.contam.f
asta

# The minimum PeptideProphet score the best peptide match of a protein mus
t have
maxIniProbTH=0.99

# The minimum PeptideProphet score a peptide must have in order to be even
 considered by Abacus
iniProbTH=0.50

# E.P.I: Experimental Peptide-probability Inclusion threshold
# If a protein does not contain at least one peptide exceeding this Peptid
eProphet score, none of the
# peptide evidence for this protein will be considered. This is applied on
 an experiment by experiment case.
epiTH=0

# The minimum ProteinProphet score a protein group must have in the COMBIN
ED file
minCombinedFilePw=0.87
```

Error running Abacus:

Fixed! interact- pep.xml files were moved to comet.pep directory along with the regular pep.xml files

```
tephra /net/nunn/vol1/home/rschauer/2021_Dec_10_Cerv
[> java -Xmx16g -jar /net/pr/vol1/ProteomicsResource/bin/abacus.jar -p Abacus_p]
arameters.txt

**********************************
        Abacus
        Version: 2.5
**********************************
Developed and written by: Damian Fermin and Alexey Nesvizhskii
Copyright 2010 Damian Fermin

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distributed under the License is distributed on an "AS IS" BASIS,
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limitations under the License.



Parameters for this execution:
        Source directory: '/net/nunn/vol1/home/rschauer/2021_Dec_10_Cerv/'
        DB name:          'ABACUSDB'
        Output file:      'outputFile'
        Combined file P:   0.9
        iniProb threshold: 0.5
        maxIniProb:        0.87
        Keep DB files:     false
        Recalc Pep Wts:    false
        Output format:     Default

Exception in thread "main" java.lang.NullPointerException
        at java.base/java.io.File.<init>(File.java:278)
        at abacus.abacus.main(abacus.java:49)
        at mainFunction.mainFunction.main(mainFunction.java:49)

tephra /net/nunn/vol1/home/rschauer/2021_Dec_10_Cerv
```
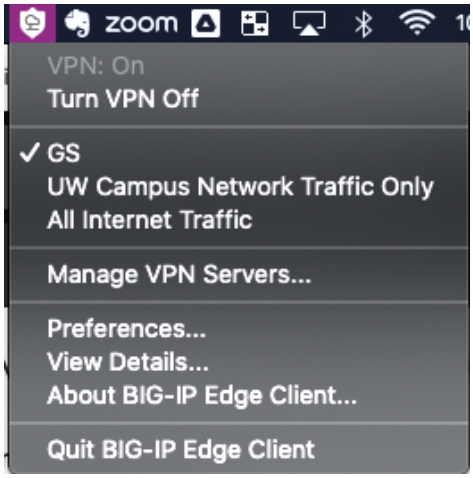
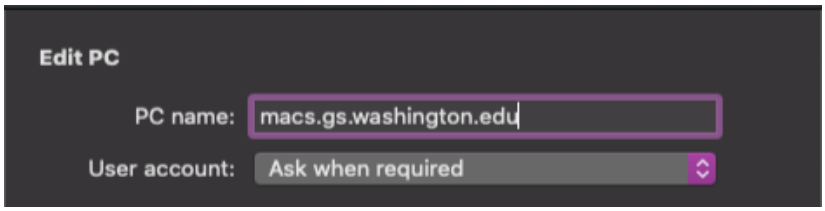

Changing Front Display Slide Show

Open BIG-IP Edge Client and log in with NetID

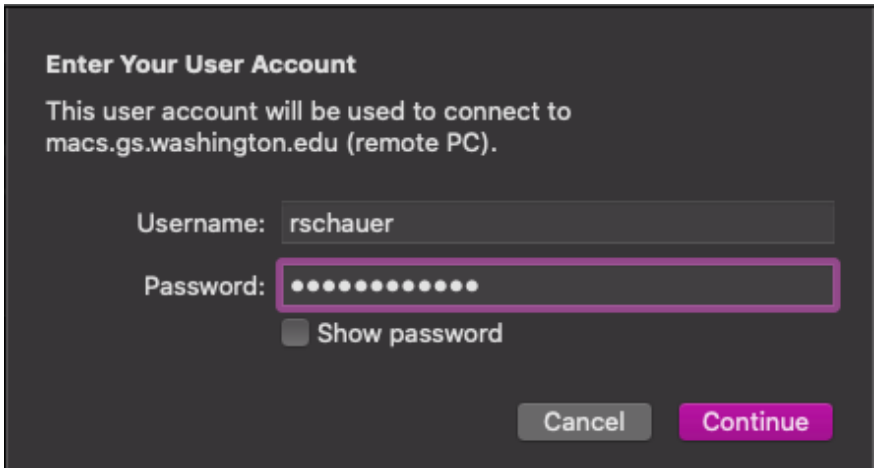
Select "GS" from menu on top ribbon



Open Microsoft Remote Desktop
Double click macs to connect



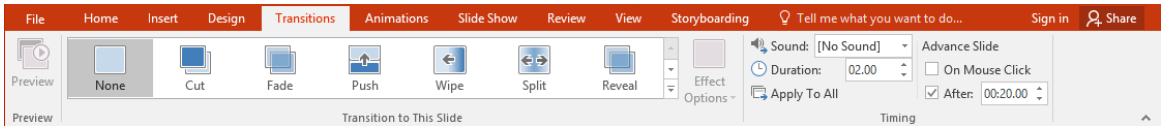
Log in with GS login



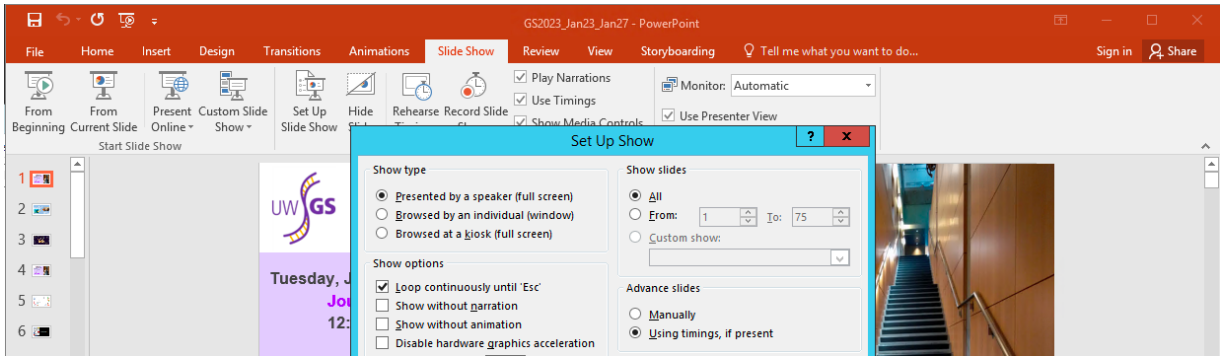
Login to Google Drive and download the powerpoint

Open powerpoint
Under Transitions set to advance slides after 20 seconds

***Make sure to click "Apply To All"!

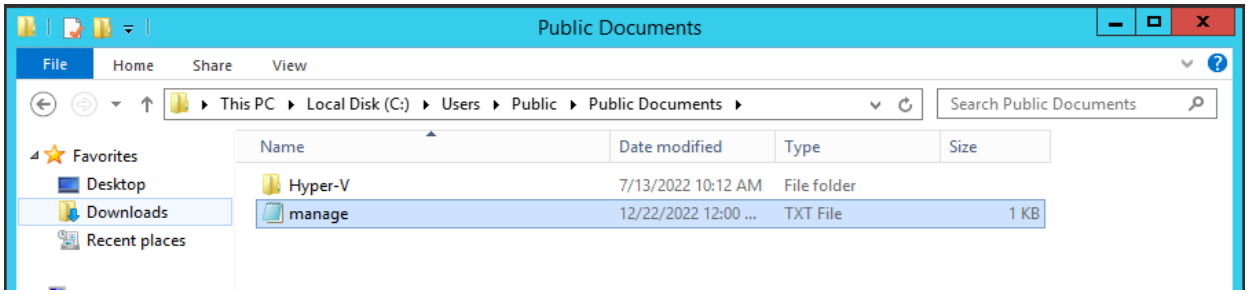


Under Slideshow select "Set Up Slide Show" then "Loop continuously until 'Esc'"

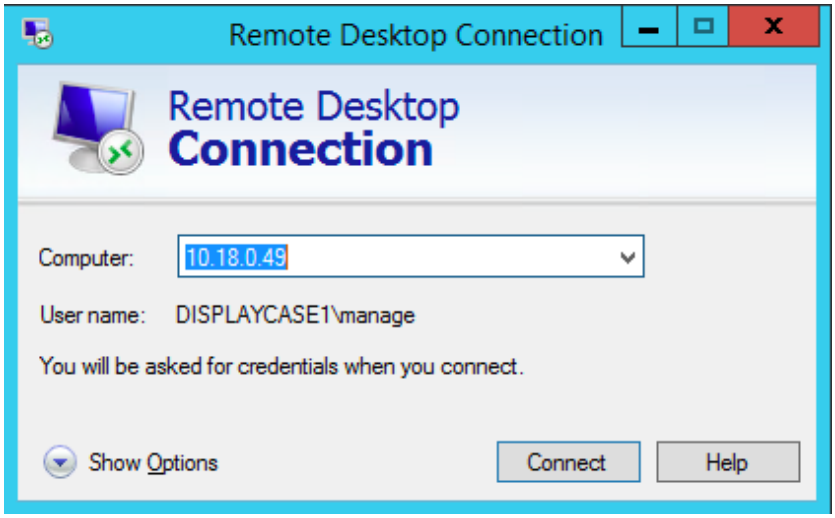


Save file as a Powerpoint Show (.pps)

Navigate to file with Front Display password and copy it

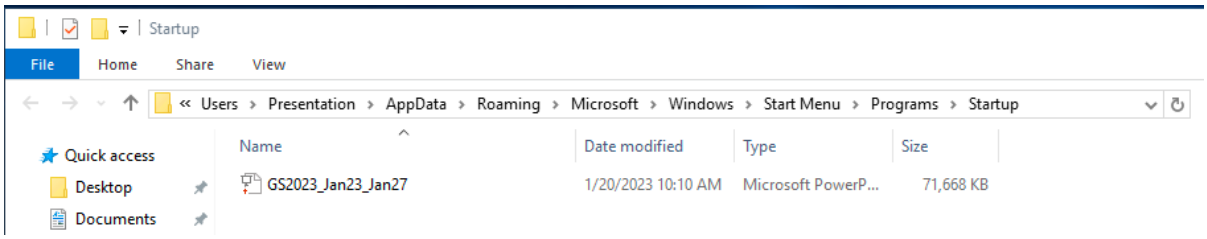


Open Remote Desktop Connection and log in with computer name and password from TXT file



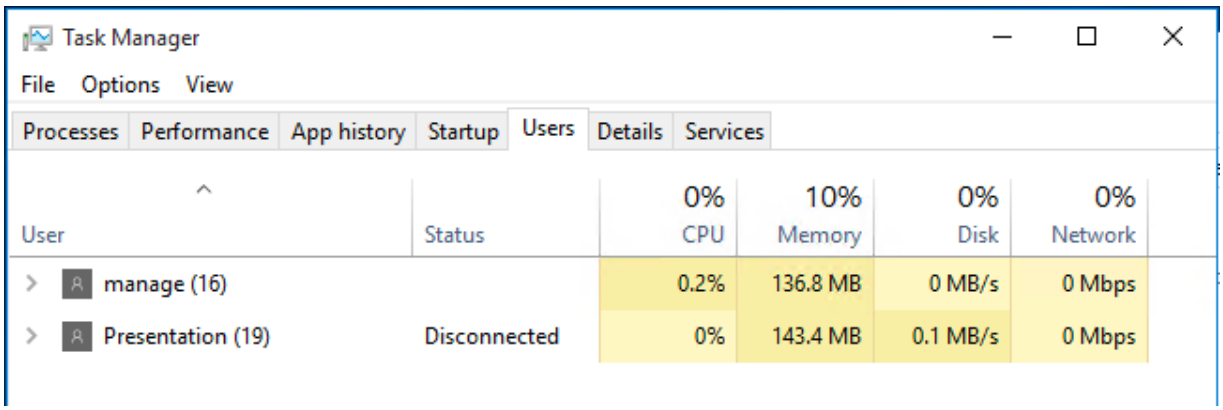
Minimize display case computer window

Copy .pps file from macs into startup folder on display case computer



Maximize display case computer window

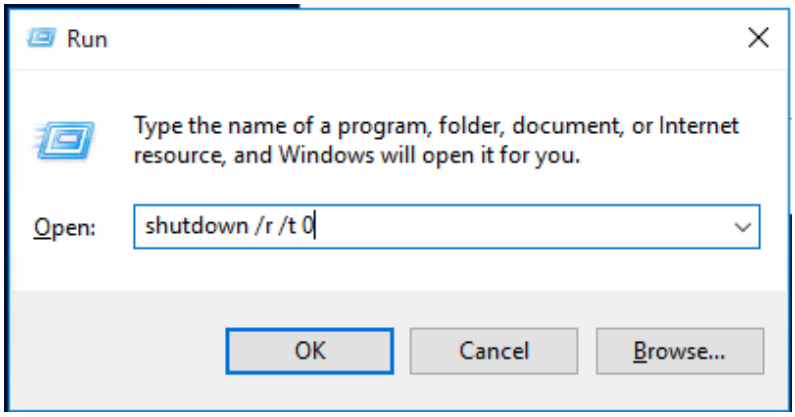
Open Task Manager and go to Users tab



Select Presentation then sign out

Move old presentation from Startup folder into old shows folder

Command+R to open run



Shutdown computer, double check display + done!

Maldonado Coral Protein Counts

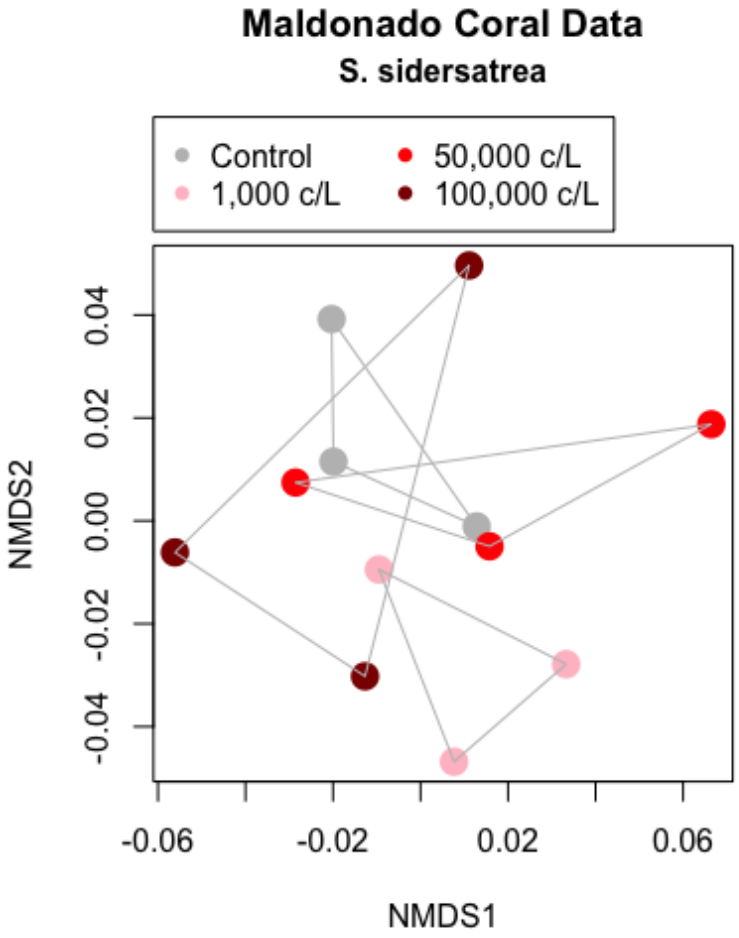
Aileen coral samples metadata.xlsx 20 kB

	A	B	C	D	E
1	File Name	Sample ID	Species	Treatment	Num. Entries
2	CORAL-31	G55 1000A	A. cervicornis	1000 c/L	1944
3	CORAL-32	G58 1000A	A. cervicornis	1000 c/L	2060
4	CORAL-44	G76 100PA	A. cervicornis	1000 c/L	2029
5	CORAL-33	G89 5000A	A. cervicornis	50000 c/L	2350
6	CORAL-34	G76 5000A	A. cervicornis	50000 c/L	2212
7	CORAL-41	G58 5000A	A. cervicornis	50000 c/L	2128
8	CORAL-29	G55 CA	A. cervicornis	Control	2019
9	CORAL-30	G76 CA	A. cervicornis	Control	2130
10	CORAL-40	G89 CA	A. cervicornis	Control	2190
11	CORAL-23	E6 1000S	S. sidersatrea	1000 c/L	1838
12	CORAL-27	A4 1000S	S. sidersatrea	1000 c/L	1462
13	CORAL-38	E10 1000S	S. sidersatrea	1000 c/L	1506
14	CORAL-25	B3 1X105S	S. sidersatrea	100000 c/L	1704
15	CORAL-37	C2 1N05S	S. sidersatrea	100000 c/L	1536
16	CORAL-36	EM1.105s?	S. sidersatrea	100000 c/L	1603
17	CORAL-24	E4 50000S	S. sidersatrea	50000 c/L	1746
18	CORAL-26	E5 5000S	S. sidersatrea	50000 c/L	1330
19	CORAL-43	E9 5000S	S. sidersatrea	50000 c/L	1568
20	CORAL-22	B6 CS	S. sidersatrea	Control	1968
21	CORAL-39	A5 CS	S. sidersatrea	Control	1609
22	CORAL-45	E1 CS	S. sidersatrea	Control	1461

https://proteomicsresource.washington.edu/net/nunn/vol1/home/rschauer
checked .prot.xml files
    Filter & Sort, Probability min: 0.9
    Filter/Sort
    File & Info
    Number of Entires Displayed

NMDS Plots:

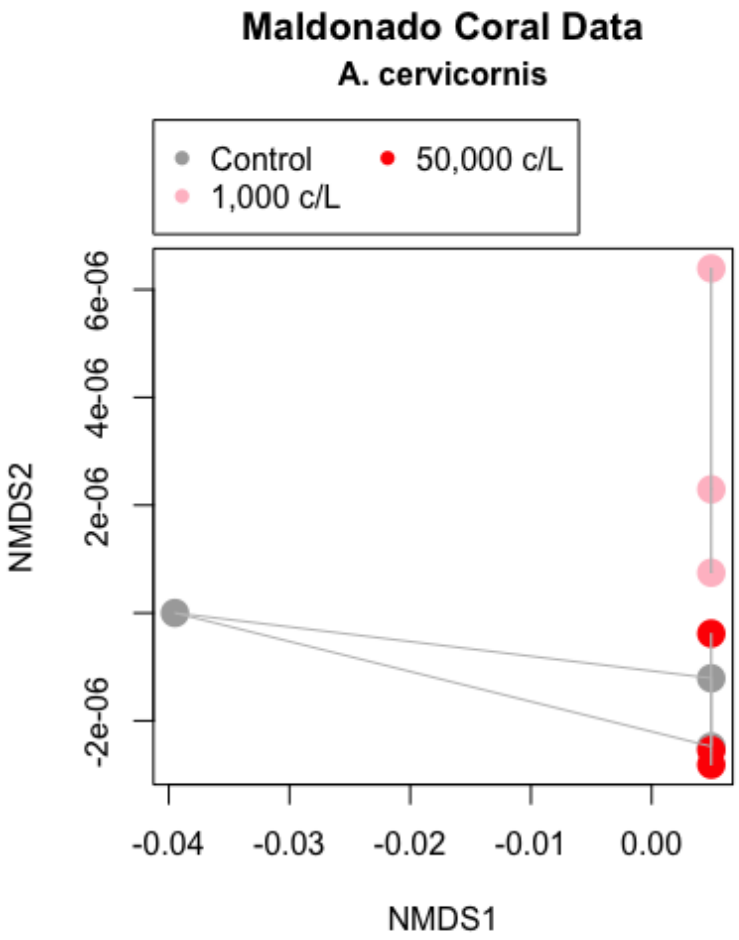
NMDS_Maldonado_Side.R 4 kB



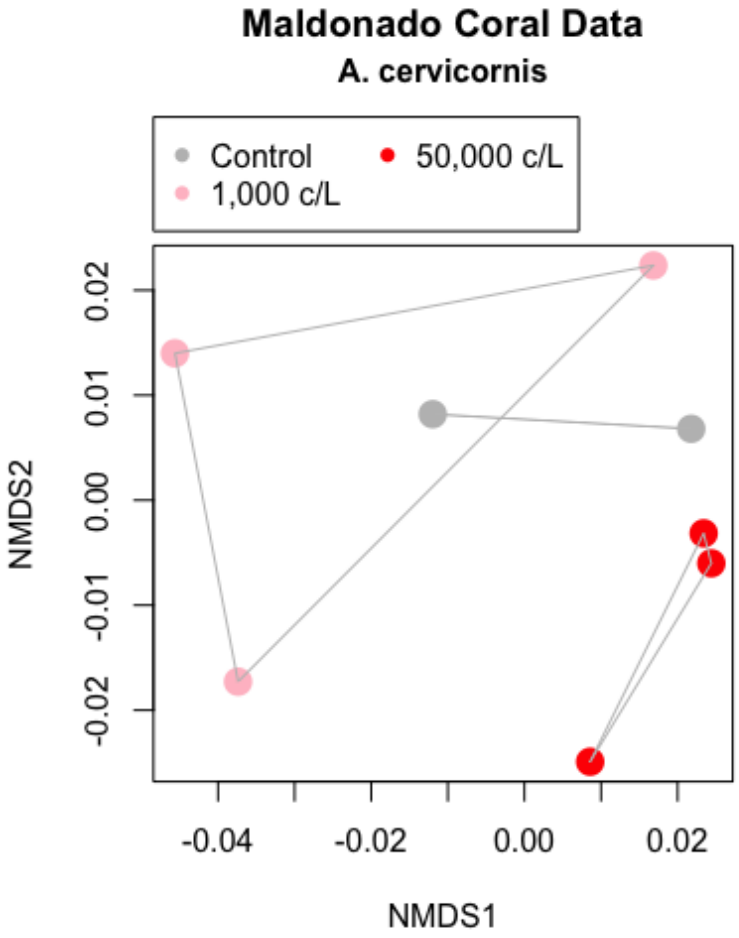
	treatment	rep	col
CORAL_22	Control	C	Grey
CORAL_23	1,000 c/L	B	Pink
CORAL_24	50,000 c/L	A	Red
CORAL_25	100,000 c/L	C	Dark Red
CORAL_26	50,000 c/L	B	Red
CORAL_27	1,000 c/L	A	Pink
CORAL_36	100,000 c/L	A	Dark Red
CORAL_37	100,000 c/L	B	Dark Red
CORAL_38	1,000 c/L	C	Pink
CORAL_39	Control	B	Grey
CORAL_43	50,000 c/L	C	Red
CORAL_45	Control	A	Grey

NMDS_Maldonado_Cerv.R 4 kB

First draft for A. cervicornis looked wonky:



Removed CORAL_29 from twopep file creation step and reran code to get new plot:



	treatment	rep	col
CORAL_30	Control	B	Grey
CORAL_31	1,000 c/L	A	Pink
CORAL_32	1,000 c/L	B	Pink
CORAL_33	50,000 c/L	C	Red
CORAL_34	50,000 c/L	B	Red
CORAL_40	Control	C	Grey
CORAL_41	50,000 c/L	A	Red
CORAL_44	1,000 c/L	C	Pink

Protein Extraction Methods Testing P. piscicida Samples

1/09/23

https://proteomicsresource.washington.edu/net/nunn/vol1/home/rschauer/2023_Jan_03_APlatt_spent/search/

Select .prot-MODELS.html file for each sample (8 total)

Sens/Error Tables

Find min_prob on Error Table closest to 0.9, record corresponding num_correct

Sample ID	min_prob	num_correct
F24a	0.9003	681
F24a King	0.9066	354
F24b	0.9035	612
F24b Micro	0.9030	918
F24b SP3	0.9004	731
F24c	0.9012	655
F24c King	0.9018	242
F24c Micro	0.9013	833

P. piscicida s-trap Analysis 1/10/23

1/10/23

9:30

Made 1000 uL SDS Buffer:

50 uL1M TEAB

250 uL20% SDS

2 uL1M MgCl2

688 uLHPLC water

10 uL100X HALT protease & phosphatase inhibitors

Vortexed

11:00

Centrifuged original samples @ 5000 g for 5 min

S24B was more viscous than others, did not completely spin down

Pipetted out supernatant (~60 - 100 uL) into new clean labelled tubes

11:45

Diluted sample

180 uLSDS Buffer

20 uLsample

12:05

Vortexed

Centrifuged diluted samples @ 5000 g for 1 min

12:17

Added standards, blanks, and samples to 96 well plate with 10 uL per well

12:45

Calculated BCA reagent volumes

200 uL * [(18 samples + 9 standards + 1 blanks) * 3] = 16,800 uL total volume

Round up to 17,000 for reagent A

17,000 uL / 50 = 340 uLreagent B

Added reagents to falcon tube and vortexed

12:54

Added 200 uL reagents to each occupied well
    Samples first, then standards

13:25
Inserted plate in Varioskan, running program with a incubation @ 37°C for 30 min before reading
Calculated volume needed for 50 ug protein in Excel

BCA_Protein_011023_APlatt.xlsx 707 kB

15:12
Centrifuged diluted samples @ 5000 g for 1 min
    Had been on ice
Aliquoted
Froze in -80

S-trap Protocol 1.12.23.pdf 2 MB

1/12/23
9:20
Thawed 40 ul sample + SDS buffer aliquots
Confirmed pH was ~8.5

9:49
Thawed 400 ug/uL enolase on ice
Diluted enolase
    Made 280 uL 100 ug/uL enolase:
        70 uL400 ug/uL enolase
        210 uLSDS buffer
        Vortexed

9:52

Added 8 uL 100 ug/uL enolase to samples
Added 0.5 uL 250 unit/uL benzonase, pipetting up and down to mix
Vortexed

10:25
Warmed @ 95°C for 10 min then cooled for 5 min at room temp
Centrifuged @ 4000 g for 30 sec to reincorporate condensate

10:27
Made S-trap binding buffer
- 2 mL1M TEAB
- 18 mLMethanol

11:02
Thawed 500 mM DTT on ice
Added 1.86 uL500 mM DTT to each sample for final concentration of 20 mM DTT

***should have been 2.02 uL
- Current sample volume = 48.5 uL
- (48.5 uL + x uL) 20 mM = 500 mM (x uL)
- 970 uL*mM + 20x uL*mM = 500x uL*mM
- 970 uL*mM = 480x uL*mM
- 2.02 uL = x

Vortexed

11:09
Warmed @ 60°C for 10 min then cooled for 5 min at room temp
Placed uncapped S-traps in clean labelled 2 mL eppie tubes for later
Centrifuged @ 4000 g for 30 sec to reincorporate condensate

11:26
Thawed 500 mM IAA
Alkylated to final 40 mM IAA
- Current sample volume = 50.36 uL
- (50.36 uL + x uL) 40 mM = 500 mM (x uL)
- 2,014.4 uL*mM + 40x uL*mM = 500x uL*mM
- 2,014.4 uL*mM = 460x uL*mM

4.38 uL = x

Added 4.38 uL500 mM IAA to each sample

Vortexed

11:32

Incubated for 30 min @ room temp in drawer

12:05

Calculated volume needed of 12% aqueous phosphoric acid for a 1:10 ratio (concentration of ~1.2%)

40 uL + 8 uL + 0.5 uL + 1.86 uL + 4.38 uL = 54.74 uL

54.74 uL * 0.12 = 6.57 uL

Added 6.57 uL 12% aqueous phosphoric acid

Vortexed

Added 350 uL S-trap binding buffer

Vortexed

12:30

Added 136 uL sample to S-traps

Centrifuged @ 4000 g for 1 min to get all solution to pass through

Dumped flow through in waste falcon tube

Rotated S-traps 180°

Repeated 2x for 3 total washes (12:44 & 12:56) adding a minute to last wash

13:07

Washed 3x with 150 uL S-trap binding buffer, rotating 180° between washes @ 4000 g for 1 min for each wash

13:28

Made 50/50 chloroform/methanol solution in fume hood with 4.5 mL of each

Rotated S-traps 180°

Added 150 uL chloroform/methanol to S-traps

Centrifuged @ 4000 g for 1 min

Dumped flow through in waste container in hood

Repeated 2x for 3 total washes

(At same time as methanol/chloroform extraction)
Made 5 mL 50 mM TEAB
    0.25 mL1M TEAB
    4.75 mLHPLC water
    Vortexed
    Kept on ice

13:53
Rotated S-traps 180°
Washed 1x with 150 uL S-trap binding buffer
    @ 7000 g for 1 min
Moved S-traps to clean labelled 2 mL eppie tubes

14:00
Added 2000 uL 50 mM TEAB vial of 100 ug Trypsin to make 0.05 ug/uL Trypsin
Added 100 uL 0.05 ug/uL Trypsin to each S-trap, making sure there were no bubbles
Loosely capped S-traps

14:10
Incubated @ 47°C for 1 hour
15:19
Centrifuged samples @ 4000 g for 30 sec to get rid of condensation
Added 80 uL 50 mM TEAB
Centrifuged spent samples @ 4000 g for 1 min
15:30
Added 80 uL 50% acetonitrile containing 0.2% formic acid to spent samples
Centrifuged spent samples @ 5000 g for 1 min
16:05
Dried down samples in speed vac (took ~ 1 hr 20 min)

17:26
Froze samples in -80

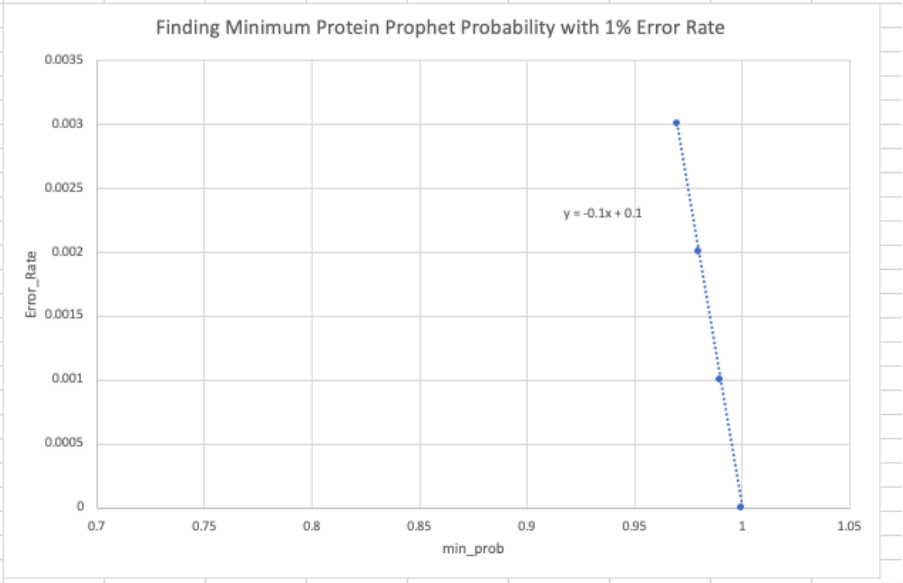
A. Platt Methods Tephra Search

1/9/23

Raw files: /net/nunn/vol1/home/rschauer/2023_Jan_03_APlatt_spent/raw
Search: /net/nunn/vol1/home/rschauer/2023_Jan_03_APlatt_spent/search
Comet.params database
/net/nunn/vol1/databases/NCBI_Pseudoalteromonas_piscicida_contam.fasta
Files:
2023_Jan_03_APlatt_spent_F24a_15
2023_Jan_03_APlatt_spent_F24a_King_35
2023_Jan_03_APlatt_spent_F24b_09
2023_Jan_03_APlatt_spent_F24b_Micro_32
2023_Jan_03_APlatt_spent_F24b_SP3_33
2023_Jan_03_APlatt_spent_F24c_28
2023_Jan_03_APlatt_spent_F24c_King_08
2023_Jan_03_APlatt_spent_F24c_Micro_34

min_prob	Error_Rate
0.97	0.003
0.98	0.002
0.99	0.001
1	0

Lin. Regression	
y	0.01
slope	-0.1
y-int	0.1
x	0.9



```
#
# ABACUS parameter file
# Generated on: 2016Jun30_1144
#

# Name to give the database
dbName=ABACUSDB

# Name of protXML file corresponding to merged/combined results
combinedFile=/net/nunn/vol1/home/rschauer/2023_Jan_03_APlatt_spent/search/in
teract-COMBINED.prot.xml

# The directory that contains the pepXML and protXML files
srcDir=/net/nunn/vol1/home/rschauer/2023_Jan_03_APlatt_spent/search/

# The name of the file where results will be saved to
outputFile=/net/nunn/vol1/home/rschauer/2023_Jan_03_APlatt_spent/search/ABAC
US_output.tsv

# The path the the FASTA formatted file used for the original protein search
# Relative paths are allowed
fasta=/net/nunn/vol1/databases/NCBI_Pseudoalteromonas_piscicida_contam.fasta

# The minimum PeptideProphet score the best peptide match of a protein must
have
maxIniProbTH=0.99

# The minimum PeptideProphet score a peptide must have in order to be even c
onsidered by Abacus
iniProbTH=0.50

# E.P.I: Experimental Peptide-probability Inclusion threshold
# If a protein does not contain at least one peptide exceeding this PeptideP
rophet score, none of the
# peptide evidence for this protein will be considered. This is applied on a
n experiment by experiment case.
epiTH=0

# The minimum ProteinProphet score a protein group must have in the COMBINED
 file
minCombinedFilePw=0.90
```

Tephra searches protocol

ssh rschauer@nexus.gs.washington.edu

Go into tephra

```
ssh tephra
```

Make a new directory to hold files

```
mkdir directory_name
```

Soft link raw data files to new directory- do this from the end destination directory and include “ .” at the end

```
ln -s path/*.raw .
```

Check all .raw files are now in your directory

```
> ls
file_1.raw
file_2.raw
file_3.raw
```

Run Comet, this creates comet.params.new

```
runCometQ --p

 single=0 all=0 wocomet=0 noprophet=0 noprotein=0 prophet0=0 noaccurate=0 expect=0
decoy=no_decoy nonparam=0 oldtpp=0 deleteraw=0 libra=0 sage=0 hours=48 numcpu=12

 Created:  comet.params.new
```

Rename Comet vim file

```
mv comet.params.new comet.params
```

Open Comet vim file

```
vim comet.params
```

Edit Comet vim file: database name and decoy search = 1, database will likely be in nunn/vol1/databases
Type "i" to enter INSERT mode
"Esc" then type ":x" to exit

```
# comet_version 2022.01 rev. 2 (f447eed)
# Comet MS/MS search engine parameters file.
# Everything following the '#' symbol is treated as a comment.
database_name =/path/contam_file.fasta
decoy_search = changeto1                       # 0=no (default), 1=internal decoy
concatenated,
```

Convert all .raw files to .mzXML files

```
convert.sh *.raw
```

Run Comet on all .mzXML files

```
runCometQ *.mzXML
```

Check you have pep.xml file for each .raw file
check status with qstat -u rschauer

```
> ls
comet.params        file_3.mzXML
file_1.mzXML        file_3.pep.xml
file_1.pep.xml      file_3.raw
file_1.raw          qsublogs
file_2.mzXML
file_2.pep.xml
file_2.raw
```

Run x-interact on all .mzXML files

```
runCometQ --wocomet --single *.mzXML
```

-OR-

Run x-interact on each file separately- make sure to change both file #s each time!
Only second has file type extension

```
xinteract -p0 -OAp -dDECOY_ -Nfile_1 file_1.pep.xml

#first name will be the prefix for the 5 interact files that will be created,
second is the file you want to run x-interact on. usually these are kept the same
```

Run Protein Prophet on all .pep.xml files

```
ProteinProphet interact*.pep.xml interact-COMBINED.prot.xml
```

Make comet.pep directory

```
mkdir comet.pep
```

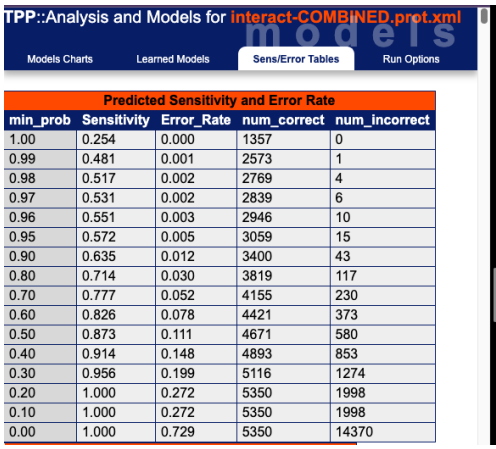
Move pep.xml files from comet search to comet.pep directory (leave interact-...pep.xml files)
Have to do individually

```
mv path/file_1.pep.xml path/comet.pep
```

Go to https://proteomicsresource.washington.edu/net/nunn/vol1/home/rschauer

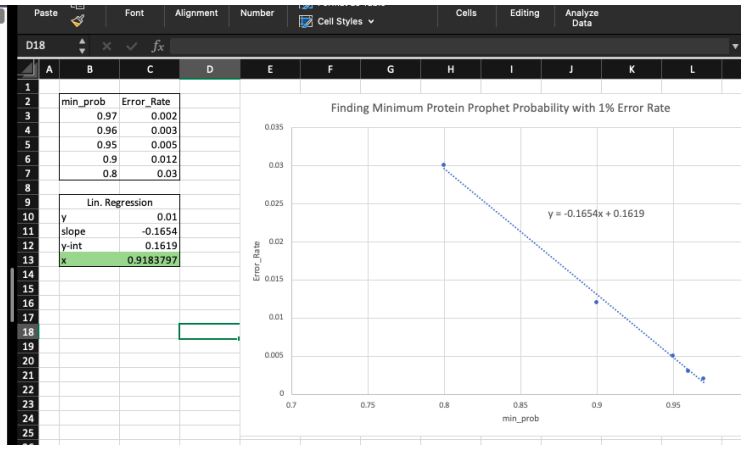
1. Log in and navigate to this project's directory
2. Open interact-COMBINED.prot.xml
3. Select "Models >"
4. Select "Sens/Error Tables" tab
5. Find the Sensitivity closest to 0.71 without going over
6. Copy the min_prob corresponding to this Sensitivity and the 4 directly above it (higher min_prob values) into an Excel file
7. Copy 5 corresponding Error_Rate values into same Excel file, creating a table with two columns
8. Plot values with min_prob on x-axis and Error_Rate on y-axis
9. Find lin regression formula
10. Plug in 0.01 for y and solve for x to get the min probability for the Abacus vim file

🔗 Calculating min Protein Prophet Score.xlsx



Predicted Sensitivity and Error Rate

min_prob	Sensitivity	Error_Rate	num_correct	num_incorrect
1.00	0.254	0.000	1357	0
0.99	0.481	0.001	2573	1
0.98	0.517	0.002	2769	4
0.97	0.531	0.002	2839	6
0.96	0.551	0.003	2946	10
0.95	0.572	0.005	3059	15
0.90	0.635	0.012	3400	43
0.80	0.714	0.030	3819	117
0.70	0.777	0.052	4155	230
0.60	0.826	0.078	4421	373
0.50	0.873	0.111	4671	580
0.40	0.914	0.148	4893	853
0.30	0.956	0.199	5116	1274
0.20	1.000	0.272	5350	1998
0.10	1.000	0.272	5350	1998
0.00	1.000	0.729	5350	14370



Copy Abacus parameters to current directory

```
cp /net/pr/vol3/www/html/protocols06/Abacus/Abacus_parameters.txt .
```

Open Abacus vim file

```
vim Abacus_parameters.txt
```

Edit Abacus vim file:

- path of protXML file
- directory w/ pepXML and protXML files
- output file name
- same path from comet vim file for .fasta
- min ProteinProphet score from Excel lin reg.

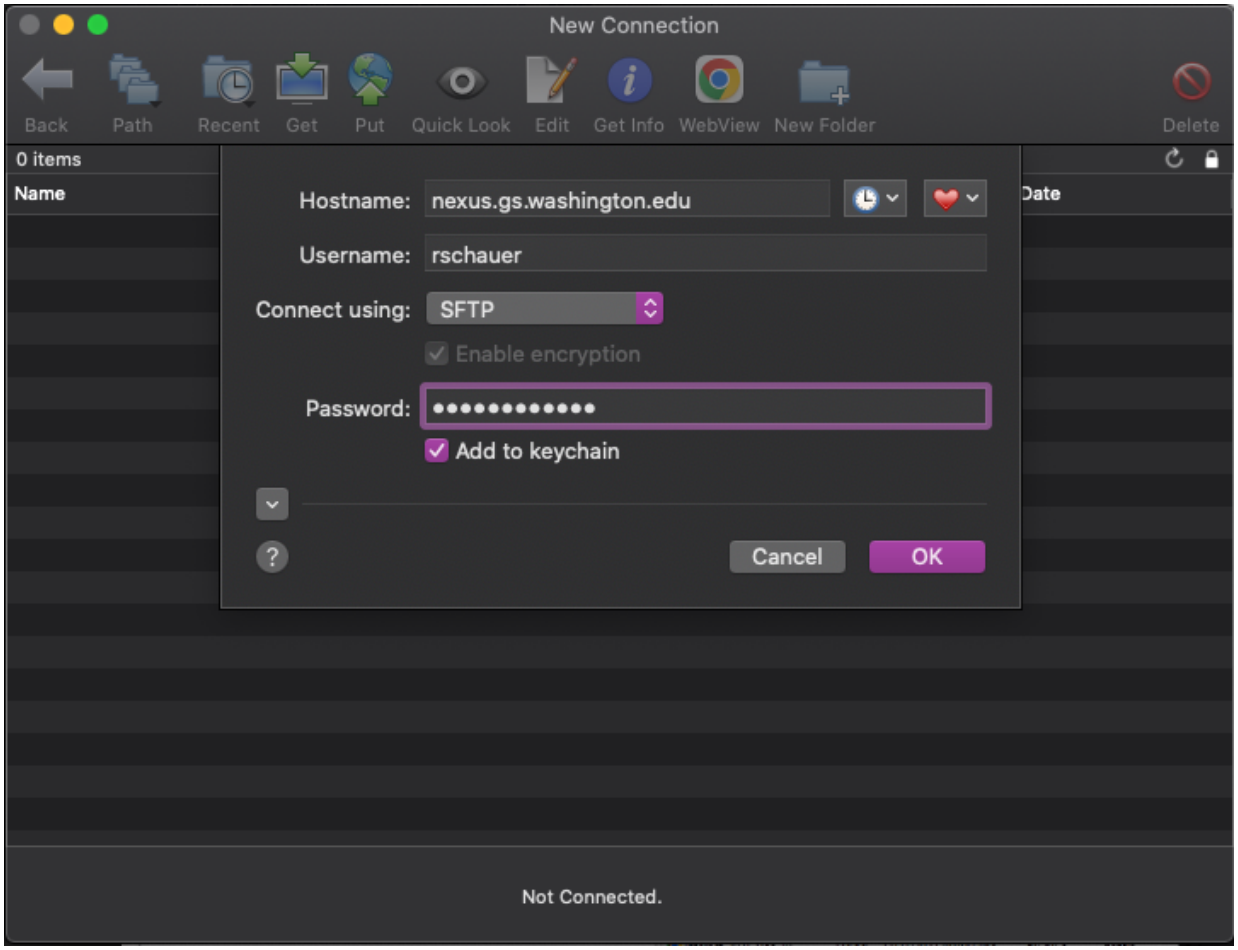
```
#Change
#
# ABACUS parameter file
# Generated on: 2016Jun30_1144
#
# Name to give the database
dbName=ABACUSDB

# Name of protXML file corresponding to merged/combined results
combinedFile=/net/nunn/vol1/home/rschauer/directory_name/interact-COMBINED.prot.xml

# The directory that contains the pepXML and protXML files
srcDir=/net/nunn/vol1/home/rschauer/directory_name

# The name of the file where results will be saved to
outputFile=/net/nunn/vol1/home/rschauer/directory_name/ABACUS_output.tsv

# The path the the FASTA formatted file used for the original protein search
# Relative paths are allowed
fasta=/path/contam_file.fasta
.
.
.
# The minimum ProteinProphet score a protein group must have in the COMBINED file
minCombinedFilePw=0.92
```

Run Abacus

https://proteomicsresource.washington.edu/protocols06/Abacus/

```
java -Xmx16g -jar /net/pr/vol1/ProteomicsResource/bin/abacus.jar -p
Abacus_parameters.txt
```

Log in to Fetch



Move ABACUS_output.tsv file to project folder in Google Drive

---

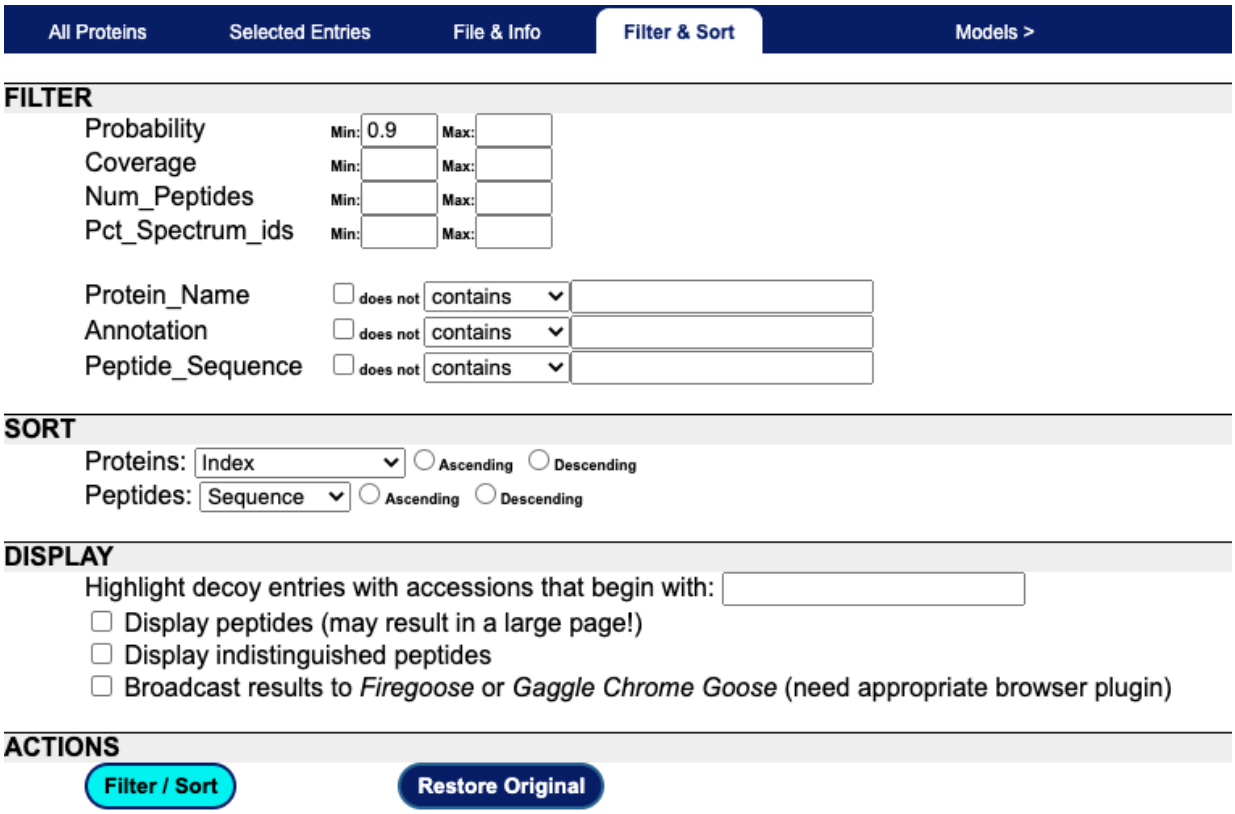
Protein Counts

Open .prot.xml files

Go to Filter & Sort tab

Enter min probability = 0.9

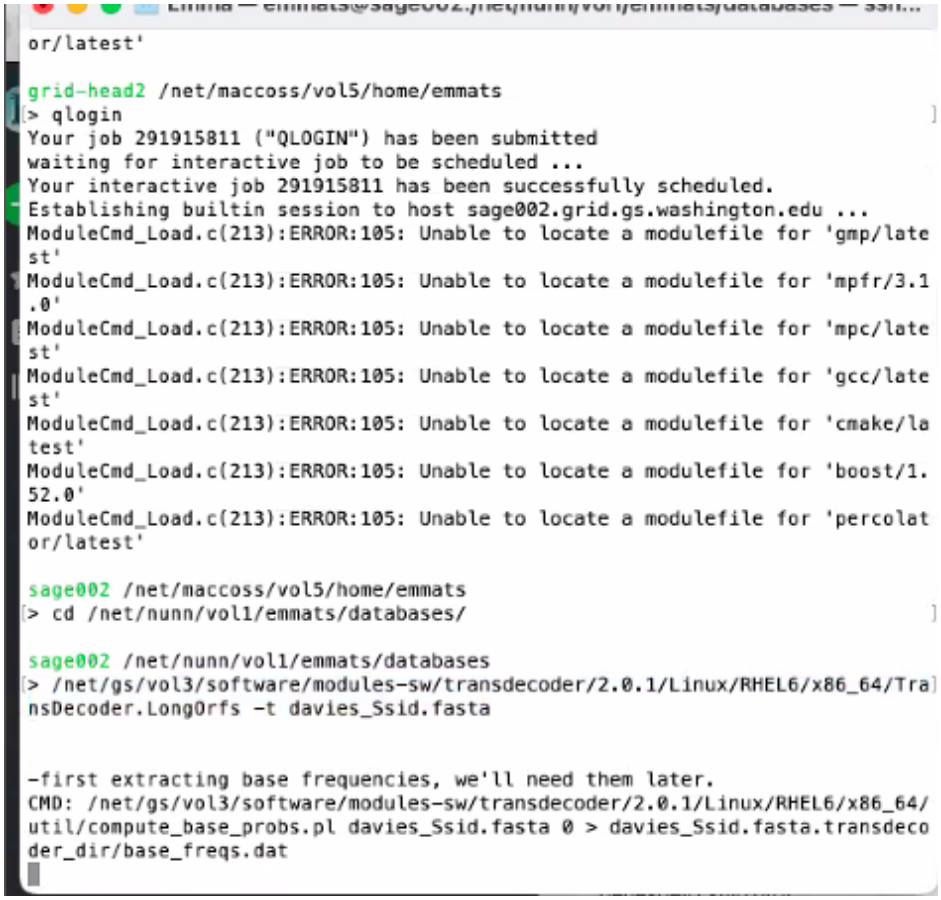
Select Filter/Sort



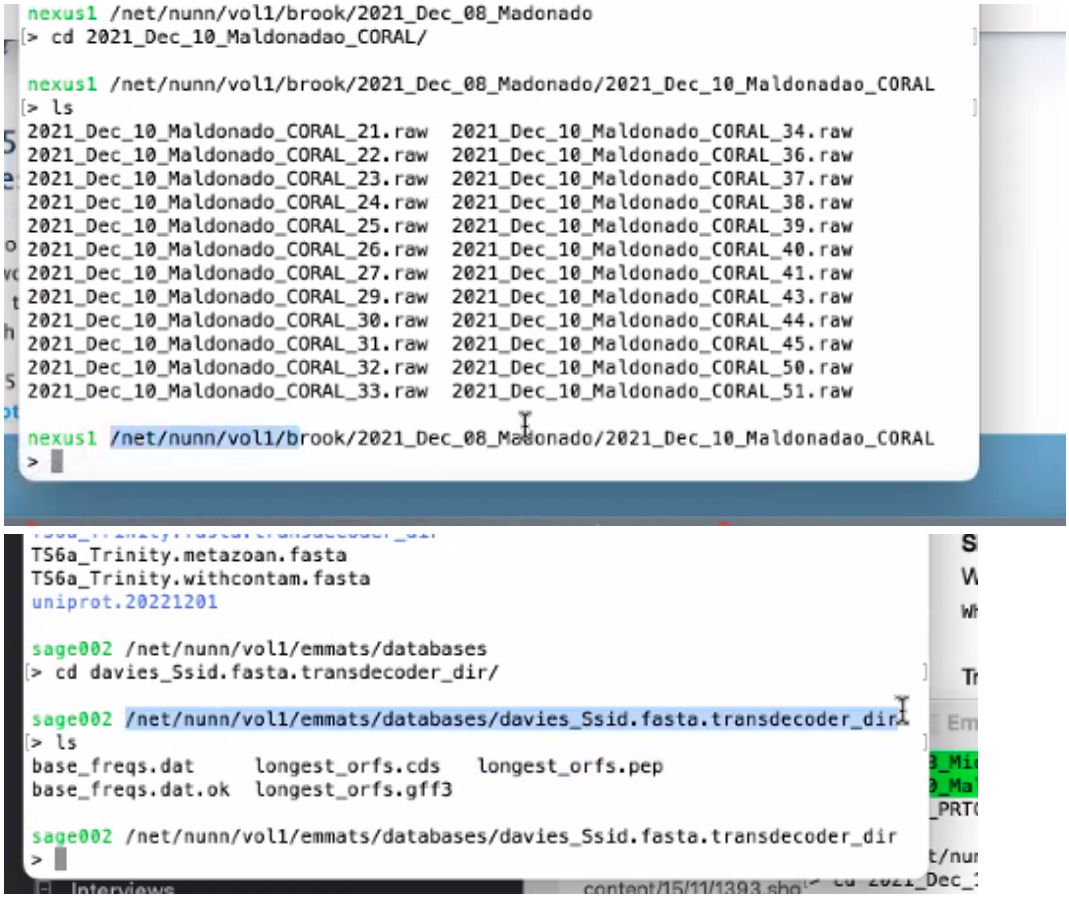
Go to File & Info tab

Record Number of entries displayed

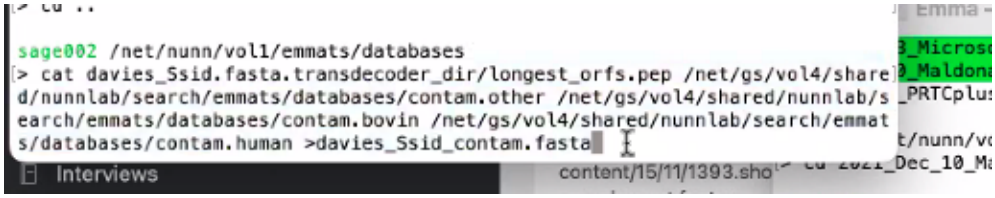
Making contams file

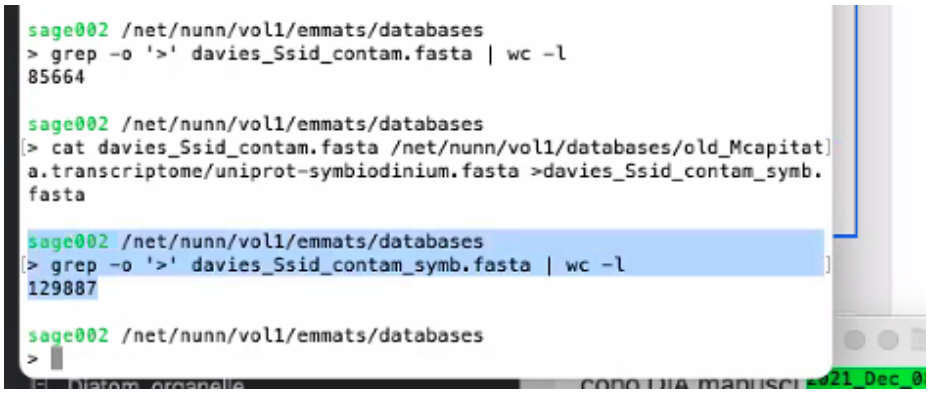

or/latest'
grid-head2 /net/maccoss/vol5/home/emmats
> qlogin
Your job 291915811 ("QLOGIN") has been submitted
waiting for interactive job to be scheduled ...
Your interactive job 291915811 has been successfully scheduled.
Establishing builtin session to host sage002.grid.gs.washington.edu ...
ModuleCmd_Load.c(213):ERROR:105: Unable to locate a modulefile for 'gmp/late
st'
ModuleCmd_Load.c(213):ERROR:105: Unable to locate a modulefile for 'mpfr/3.1
.0'
ModuleCmd_Load.c(213):ERROR:105: Unable to locate a modulefile for 'mpc/late
st'
ModuleCmd_Load.c(213):ERROR:105: Unable to locate a modulefile for 'gcc/late
st'
ModuleCmd_Load.c(213):ERROR:105: Unable to locate a modulefile for 'cmake/la
test'
ModuleCmd_Load.c(213):ERROR:105: Unable to locate a modulefile for 'boost/1.
52.0'
ModuleCmd_Load.c(213):ERROR:105: Unable to locate a modulefile for 'percolat
or/latest'
sage002 /net/maccoss/vol5/home/emmats
> cd /net/nunn/vol1/emmats/databases/
sage002 /net/nunn/vol1/emmats/databases
> /net/gs/vol3/software/modules-sw/transdecoder/2.0.1/Linux/RHEL6/x86_64/Tra
nsDecoder.LongOrfs -t davies_Ssid.fasta
-first extracting base frequencies, we'll need them later.
CMD: /net/gs/vol3/software/modules-sw/transdecoder/2.0.1/Linux/RHEL6/x86_64/
util/compute_base_probs.pl davies_Ssid.fasta 0 > davies_Ssid.fasta.transdeco
der_dir/base_freqs.dat

raw files:



wand longest_orfs.pep (this adds contaminents)





^adding symbionts to contam file

Linux commands

ssh rschauer@nexus.gs.washington.edu

cat	combine two files
cd	navigate to a directory
cd ..	move up a directory, add /.. for each directory you want to move
ls	show directory contents
mkdir *directoryname*	make new directory
mv *filename newpath/filename*	move a file
mv *oldname newname*	rename a file
rm *directoryname*	remove an empty directory
rm *filename*	remove file (type "y" after)
rm -rf *directoryname*	remove a directory with contents
tab	auto complete file or directory name
qstat -u rschauer	check status of requests (ie after running comet)
up arrow	scroll through previous commands

Proteomics Intro

Proteins

catalysts, structural integrity, adhesion, cell signaling

Keratin is common background detected my mass spec
Average in ~300 amino acids

Proteomic analysis tells you:
taxonomy an function
cross-linked proteins
cellular location

All amino acids have a carboxyl and amine group, these link to make covalent peptide bod that can break with acid
separated with chromatography

Bottom-up analysis
easier to measure individual peptides vs whole proteins
smaller range of pos. charges to look for

Sample prep
Lyse protein with enzyme (we use Trypsin)
separate with HPLCfa
separates components in mixture
Every bead had string of Carbon coming off
send different liquids through, components come off with what they are more soluble in
when they come off is retention time
goes 2% acn (organic) to 35% scn
longer the gradient length, longer the retention time (we usually do 90 min)
needs to be ionized after chromatography
attach pos Hydrogen ions as peptides are sprayed off of column needle
these then go into mass spec

Mass Spec
We use Orbitrap usually
resolution = mass/peak width (high res have smaller peak width)

Data Dependent Analysis looks at most abundant ions, big picture, minimal sample needed

Single

Spectral counting gives you relative abundance of a peptide
p. piscacida will be DDA analysis

Comet gives you hypothetical mass/charge then matches to your actual to find most likely

Kingfisher SP3 Protocol

Word version:

Kingfisher SP3 Protocol.docx 20 kB

Volume
Reagent
Time & Temp
Calculations

DAY 1
Tissue Lysis

1. CALCULATE : Sample # * 120 uL = Total volume needed
    - For 1,000 uL:
        - 50 uL 1M TEAB (cold room)
        - 250 uL 20% SDS (bench)
        - 2 uL 1 M MgCl2 (bench)
        - 688 uL HPLC (above bench)
        - 10 uL 100X HALT protease & phosphatase inhibitors (cold room, come in 20uL tubes)
        - Vortex
2. Defrost samples on ice & immediately spin in large centrifuge @ 4°C and 10,000g for 10 min to pellet the cells
3. Sonication
    1. Add 120 uL SDS Buffer to each sample

    2. To cool: use ice bucket OR get small beaker and add approx. 20 uL ethanol (bench and next to sonicator), add dry ice pellets one at a time after each one is dissolved
    3. Sonicate at speed 3 for 20 sec then hold in ice for 30 sec OR cold ethanol for 15 sec, 3 times per sample
    4. Clean probe with ethanol in between samples
4. Can freeze in -80 or continue to BCA

BCA Protein Assay

1. CALCULATE :
    1. (Sample # + 27 standards) * 200 uL (round up to nearest multiple of 500) = Volume Reagent A
    2. (Volume Reagent A / 500) * 10 = Volume Reagent B
    3. Double check 50:1 ratio for Reagent A:Reagent B; multiplying volume Reagent B * 50 should equal volume Reagent A
2. Create BCA standards according to manufacturer's instructions if not already made
3. Add Reagent A + Reagent B to a falcon tube and vortex (BCA box above bench)
4. Add 10 uL standards along top 3 rows of the plate with replicates in columns
5. Add 10 uL samples along next 3 rows of the plate with replicates in columns
6. Add 200 uL Reagent solution to every occupied well and gently shake
7. Heat plate @ 37°C for 30 min (hallway)
8. Read plate using Varioskan
    1. Turn on machine then log into computer, click icon w/o box in upper left
    2. Click your folder then an old session, immediately save as with new name
    3. Adjust plate layout
    4. Insert plate into machine and run session, take picture of results!
    5. Save report to a USB
9. Fill out BCA Excel sheet to find sample protein concentrations in ug/mL and uL volume needed for 50 ug of protein
10. Aliquot 50 ug protein for each sample in 1.5 mL tubes
11. Add SDS buffer so each sample has 40 uL total volume
12. Freeze in -80

DAY 2

Sample Preparation

1. Aliquot 50 ug protein for each sample in 1.5 mL Eppendorf tubes
2. Make 2% SDS lysis buffer
    - For 1000 uL:
        - 2 uL 400 ng/uL enolase (-80 C3 drawer)
        - 50 uL 1M TEAB (cold room)
        - 100 uL 20% SDS (bench)
        - 842 uL HPLC water (bench)
    - Vortex
3. Add SDS buffer so each tube has 200 uL total volume

Reduction & Alkylation

1. Add 500 mM TCEP (Rich's bench) to final concentration of 10 mM
    - CALCULATE: 500 mM * x = 10 mM (current vol. + x) where x = volume TCEP
    - For 200 uL starting samples add 4.1 uL
    - Vortex
2. Heat at 37°C for 1 hour
    - Let tubes cool completely
3. Add 500 mM IAA (R2-D2 freezer, keep in a dark place) to final concentration of 15 mM
    - CALCULATE: 500 mM * x = 15 mM (current vol. + x) where x = volume IAA
    - For 200 uL starting samples add 6.3 uL
    - Pipette up and down gently to mix
4. Incubate for 30 min in the dark at room temp
5. Add 500 mM DTT (R2-D2 freezer) to final concentration of 10 mM to inactivate free IAA
    - CALCULATE: 500 mM * x = 10 mM (current vol. + x) where x = volume DTT
    - For 200 uL starting samples add 4.3 uL
6. Let sit for 15 min at room temp

Wash & Digestion Plate Preparation

NOTE: For volumes 50 uL - 150 uL use shallow well plates, for volumes 150 uL - 1000 uL use deep well plates

1. Make 3 95% ACN (bench) deep well wash plates
    - 1 mL well for each sample
    - Label both the plate and the lid with Solution and Wash #
2. Make 2 70% EtOH (bench) deep well wash plates

- 1 mL well for each sample
- Label both the plate and the lid with Solution and Wash #

CALCULATIONS NOTE: calculating how much (x) of initial concentration (Ci) to add to create new final concentration (Cf) at a specific final volume (Vf). Make sure units match!!

Cix=Cf(Vf+x)
Cix=CfVf+Cfx
Cix-Cfx=CfVf
Ci-fx=CfVf
x= CfVfCi-f

Protein Precipitation

1. Add full samples to deep well plate, with each in a separate well
   - Cross off wells you are using (can only be used once)
   - Label both the plate and the lid with " Protein Precipitation"
2. Add 12.5 uL of the MagReSyn Hydroxyl beads (R4 fridge 1.5 mL & 2 mL aliquots)
   - ~5-10 um; stock concentration of 20 ug/uL in 20% EtOH
   - Pool all beads together before adding
   - Pipette mix gently
   - ONCE BEADS ARE ADDED YOU CANNOT FREEZE OR VORTEX
3. Add 100% ACN (bench) to final concentration of 70% to precipitate proteins
   - CALCULATE: 100 * x = 70 (current vol. + x) where x = volume ACN
   - For 200 uL starting samples add 530.1 uL
   - Pipette mix gently
4. Let sit 10 min at room temp

Operating Kingfisher & Adding Plates

1. Turn on computer (should already be on) and open up "Bindit 3.2 Kingfisher Program"
2. Username is "admin" (should autofill) and no password
3. Click "Open" to open Protocol Manager
4. Open a previous protocol, should have prefix: SP3_hydroxlyl_60min_SDS_LongCollection_RJ_CCW
5. Save as and rename with initials and date in Nunn folder
6. Go to layout to double check the capture volumes (should be accurate)
   - Sample volume (enter final volume after 100% ACN was added)
     - 757 uL for 200 uL starting samples

    - ACN volume (final volume you want elutions to be- this will be vol. AmBic + Trypsin you add to each well in Digestion section)
        - Should be >150 uL, some is lost to evaporation
        - 150 uL with give you final protein concentration of ~0.33 ug/uL
7. Click Start
8. Add plates in order shown on Kingfisher instrument screen, pushing start after each one
    - First will be the tip plate kept in the shallow holder
    - Skip elution 1 - just press start to go to the next one.
    - Make sure to match A1 on plate to A1 on instrument
9. Once all of the plates are loaded press Start
    - You will have ~35 min before adding Trypsin

Digestion

1. If not using bench top stock: Make 50 mM AmBic (keep on ice)
    - For 1 mL:
        - 1 mL HPLC water (bench)
        - 4 mg AmBic (above large centrifuge)
    - Vortex
2. @ ~1h17m make 1 ug/uL Trypsin
    - Add 20 uL 50 mM AmBic to 20 ug Trypsin vial
    - Vortex
3. Make Trypsin solution (Want 20:1 (protein:Trypsin))
    - 150 mL for each sample: Multiply by sample # + 1 so you have extra!
        - 2.5 uL 1 ug/uL Trypsin
        - 147.5 uL 50 mM AmBic
    - Vortex
4. Add 150 uL to each well
5. Add the Trypsin plate @ 1h12m
    - MAKE SURE TO ADD PLATE RIGHT AFTER INSTRUMENT PAUSES OR BEADS WILL DRY OUT
6. Click start

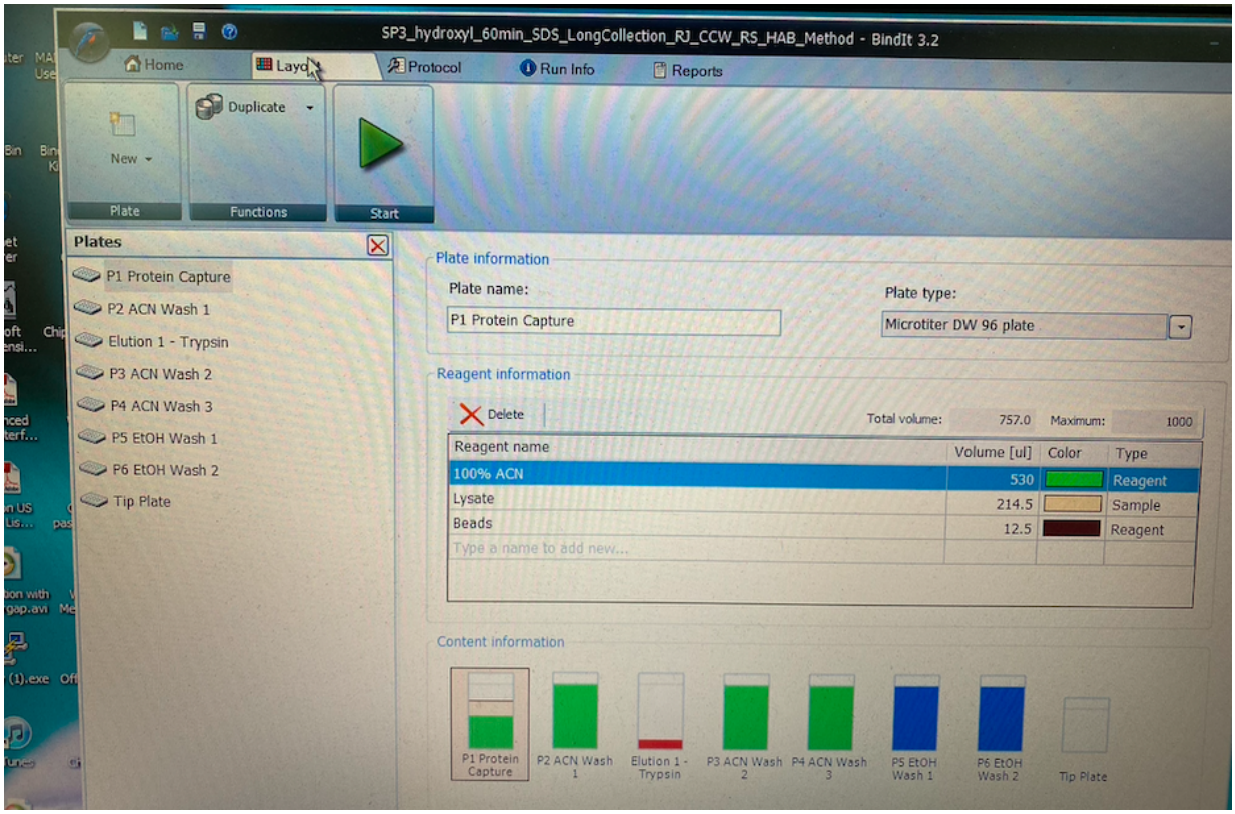
Quenching the Digestion

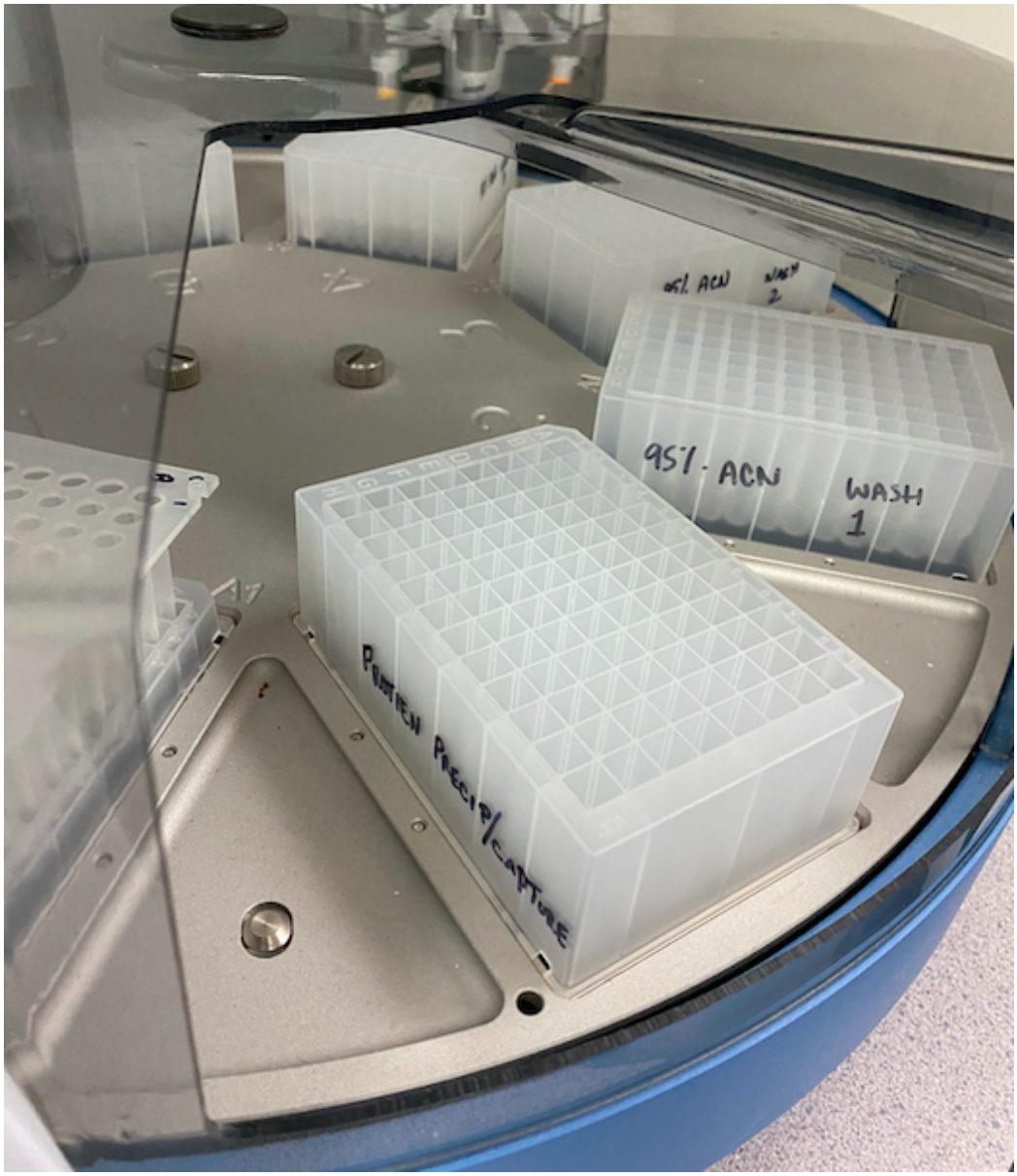
1. Before Instrument is done:
    - Label new eppie tubes for each sample

    - Check you have 100% Formic Acid (under hood)
    - Place the formic acid, pipettes, and pipette tips in the hood
2. When instrument is done
    - Will produce a pdf summary, exit out of it
    - Remove all of the plates in the order that the program indicates
    - Bring Trypsin plate to hood
3. Add 100% Formic Acid to final concentration of 5%
    - CALCULATE: 100 * x = 5 (current vol. + x) where x = volume Formic Acid
    - For 150 uL elutions add 7.89 uL
    - Pipette mix gently
4. Transfer samples to the newly labeled eppie tubes

Resuspension

1. Add 0.1% Formic Acid (bench) to 0.5 ug/uL
    - For 50 ug starting protein add 100 uL
2. Shake at 1,400 @ 37°C for 10 min
3. Freeze in -80
4. Later, before running on mass spec:
    1. Spin down and aliquot supernatant
    2. Add PRTC


SP3_hydroxyl_60min_SDS_LongCollection_RJ_CCW_RS_HAB_Method - BindIt 3.2
Home
Layout
Protocol
Run Info
Reports
Duplicate
New
Plate
Functions
Start
Plates
P1 Protein Capture
P2 ACN Wash 1
Elution 1 - Trypsin
P3 ACN Wash 2
P4 ACN Wash 3
P5 EtOH Wash 1
P6 EtOH Wash 2
Tip Plate
Plate information
Plate name:
P1 Protein Capture
Plate type:
Microtiter DW 96 plate
Reagent information
Delete
Total volume: 757.0
Maximum: 1000
Reagent name
Volume [ul]
Color
Type
100% ACN 530 Reagent
Lysate 214.5 Sample
Beads 12.5 Reagent
Type a name to add new...
Content information
P1 Protein Capture
P2 ACN Wash 1
Elution 1 - Trypsin
P3 ACN Wash 2
P4 ACN Wash 3
P5 EtOH Wash 1
P6 EtOH Wash 2
Tip Plate



S-trap Analysis 12/8/22

18 total samples:
P. piscicida

	1 hr A	1 hr B	1 hr C	8 hr A	8 hr B	8 hr C	24 hr A	24 hr B	24 hr C

Spen t	S1A	S1B	S1C	S8A	S8B	S8C	S24A	S24B	S24C
f/2	f/2 1A	f/2 1B	f/2 1C	f/2 8A	f/2 8B	f/2 8C	f/2 24A	f/2 24B	f/2 24C

11/21/22
13:00
Pelleted cells by defrosting in ice and spinning @ 4°C and 10,000g for 10 min

14:00
Calculated SDS buffer volume:
    18 samples * 120 uL = 2,160 uL; will need to multiple 1,000 uL recipe x3
Made 3,000 uLSDS buffer:
    50 uL1M TEAB * 3 = 150 uL
    250 uL20% SDS * 3 = 750 uL
    2 uL1 M MgCl2 * 3 = 6 uL
    688 uLHPLC * 3 = 2,064 uL
    10 uL100X HALTprotease & phosphatase inhibitors * 3 = 30 uL
Vortexed

14:45
Sonicated each sample @ speed 3 for 20 sec then in ethanol + dry ice for 15 sec x3

15:55
Froze samples in -80

11/22/22
13:00
Calculated BCA reagent volumes
    200 uL * [(18 samples + 9 standards) * 3] = 16,200 uL total volume
    Round up to 17,000 for reagent A
    17,000 uL / 50 = 340 uLreagent B

Divided into two plates: one for spent, one for f/2. Each has triplicates of samples and standards.

- Made more BCA reagent solution for second plate's standards: 200 uL * (9 standards * 3) = 5,400 uL
- Round up to 6,000 for reagent A
- 6,000 uL / 50 = 120 uLreagent B

Tested one well with 10 uL of sample + 200 uL reagent solution - Too purple! need to dilute 10:1

- Added 200 uL SDS Buffer + 20 uL sample to clean labelled 1.5 mL eppie tubes, vortexed
  - original green labels -> diluted red labels
  - original yellow labels -> diluted purple labels
- 200 uL * 18 = 3,600 uL -> rounded to 4,000 uL
- Made 4,000 uLSDS buffer
  - 50 uL1M TEAB * 4 = 200 uL
  - 250 uL20% SDS * 4 = 1,000 uL
  - 2 uL1 M MgCl2 * 4 = 8 uL
  - 688 uLHPLC * 4 = 2,752 uL
  - 10 uL100X HALTprotease & phosphatase inhibitors * 4 = 40 uL

14:06
Warmed plates @ 37°C for 30 min
14:36
Ran BCA and recorded outputs in Excel

BCA_Protein_112222_f2.xlsx

BCA_Protein_112222_spent.xlsx

11/29/22

Calculated how much protein lysate volume to aliquot for 50 ug protein

- Graphed Standards vs Concentration to get scatter plot w/ line of best fit
- Used equation from plot with highest R-sq value and solved for sample concentrations (x) by plugging in BCA output (y)

Averaged triplicates' concentrations (ug/mL) and divided by 1000 to convert to ug/uL
Divided 50 ug by sample concentration (ug/uL) to get volume needed
Subtracted volume from 40 uL to get volume of SDS buffer needed to have total volume of 40 uL

S-traps 113022.xlsx

Sample	Average Concentration Undiluted (ug/uL)	Undiluted Protein lysate vol. needed for 50 ug S-Trap (uL): 50 / Conc.	SDS buffer needed for 40 uL total		Sample	Average Concentration Undiluted (ug/uL)	Undiluted Protein lysate vol. needed for 50 ug S-Trap (uL): 50 / Conc.	SDS buffer needed for 40 uL total
f/2 1A	2.086	23.97	16.03		s 1A	1.893	26.41	13.59
f/2 1B	2.154	23.22	16.78		s 1B	1.942	25.74	14.26
f/2 1C	2.059	24.28	15.72		s 1C	2.011	24.86	15.14
f/2 8A	2.000	25.00	15.00		s 8A	1.871	26.73	13.27
f/2 8B	1.791	27.92	12.08		s 8B	1.804	27.71	12.29
f/2 8C	2.052	24.37	15.63		s 8C	1.820	27.48	12.52

f/2 24B	2.172	23.02	16.98		s 24B	1.427	35.05	4.95
f/2 24C	1.782	28.06	11.94		s 24C	2.059	24.28	15.72

Froze aliquoted samples in -80

12/8/22
9:17
Thawed undiluted samples

9:30
Made 500 uL SDS Buffer:
- 25 uL1M TEAB
- 125 uL20% SDS
- 1 uL1M MgCl2
- 344 uLHPLC water
- 5 uL100X HALT protease & phosphatase inhibitors
- Vortexed

9:40
Pipetted undiluted sample and SDS buffer into new labelled 1.5 mL eppie tubes according to above table to have 50 ug of protein and a final volume of 40 uL per sample
Vortexed
Confirmed pH was ~7

10:22
Thawed 400 ug/uL enolase on ice
Diluted enolase
- Made 280 uL 100 ug/uL enolase:
  - 175 uLSDS buffer
  - 35 uLHPLC water

70 uL400 ug/uL enolase
Vortexed

10:28
Added 8 uL 100 ug/uL enolase to samples

10:38
Added 0.5 uL 250 unit/uL benzonase, pipetting up and down to mix
Vortexed

10:48
Warmed @ 95°C for 10 min then cooled for 5 min at room temp
Centrifuged @ 4000 g for 30 sec to reincorporate condensate

11:11
Thawed 500 mM DTT on ice
Reduced 500 mM DTT to 20 mM DTT
Current sample volume = 48.5 uL
48.5 uL / (500 mM/20 mM) = 1.94 uL
Added 1.94 uL20 mM DTT to each sample
Vortexed

11:20
Warmed @ 60°C for 10 min then cooled for 5 min at room temp
Centrifuged @ 4000 g for 30 sec to reincorporate condensate

11:23
Made 5 mL 50 mM TEAB
0.25 mL1M TEAB
4.75 mLHPLC water
Vortexed

11:41
Thawed 500 mM IAA
Alkylated 500 mM IAA to 40 mM IAA
Current sample volume = 50.44 uL

50.44 uL / (500 mM/40 mM) = 4.04 uL
Added 4.04 uL40 mM IAA to each sample
Vortexed

11:47
Incubated for 30 min @ room temp in drawer

12:20
Calculated volume needed of 12% aqueous phosphoric acid for a 1:10 ratio (concentration of ~1.2%)
40 uL + 8 uL + 0.5 uL + 1.94 uL + 4.04 uL = 54.48 uL
54.48 uL * 0.12 = 6.54 uL
Added 6.4 uL 12% aqueous phosphoric acid
Vortexed
Confirmed pH was ~1

12:30
Made S-trap binding buffer
2 mL1M TEAB
18 mLMethanol
Added 350 uL S-trap binding buffer
Vortexed
Placed uncapped S-traps in clean labelled 1.5 mL eppie tubes

12:55
Added 136 uL sample to S-traps

13:12
Centrifuged @ 4000 g for 1 min to get all solution to pass through
Dumped flow through in waste falcon tube

13:34
Rotated S-traps 180°
Added 136 uL sample to S-traps
Centrifuged @ 5000 g for 2 min to get all solution to pass through
Dumped flow through in waste falcon tube

13:50
Rotated S-traps 180°
Added remaining sample to S-traps
Centrifuged @ 6000 g for 2 min to get all solution to pass through
    Dumped flow through in waste falcon tube

14:02
Washed 3x with 150 uL S-trap binding buffer, rotating 180° between washes
    @ 8000 g for 1 min
14:19
Made 50/50 chloroform/methanol solution in fume hood with 5 mL of each
Rotated S-traps 180°
Added 150 uL chloroform/methanol to S-traps
Centrifuged @ 7000 g for 1 min
    Dumped flow through in waste container in hood
Repeated 2x for 3 total washes (14:30 & 14:37)

14:53
Rotated S-traps 180°
Washed 1x with 150 uL S-trap binding buffer
    @ 7000 g for 1 min

15:06
Moved S-traps to clean labelled 2 mL eppie tubes

15:08
Added 2000 uL 50 mM TEAB each to vial of 100 ug Trypsin to make 0.05 ug/uL Trypsin
Added 100 uL 0.05 ug/uL Trypsin to each S-trap, making sure there were no bubbles
Loosely capped S-traps

15:19
Incubated @ 47°C for 1 hour
16:23
Centrifuged samples @ 4000 g for 30 sec to get rid of condensation

Added 80 uL 50 mM TEAB
Centrifuged spent samples @ 5000 g for 1 min
16:32
Added 80 uL 50% acetonitrile containing 0.2% formic acid to spent samples
Centrifuged spent samples @ 5000 g for 1 min
16:40
Froze elutions in -80

12/15/22
13:49
Dried down thawed elutions in speed vac
Temp: none, heat time: 2 hrs, vac pressure: 1
Total time: 1h 6m

14:55
Removed dried samples from speed vac and froze in -80

S-trap Analysis 11/21/22 - 12/1/22

18 total samples:
P. piscicida

	1 hr A	1 hr B	1 hr C	8 hr A	8 hr B	8 hr C	24 hr A	24 hr B	24 hr C
Spent	S1A	S1B	S1C	S8A	S8B	S8C	S24A	S24B	S24C
f/2	f/2 1A	f/2 1B	f/2 1C	f/2 8A	f/2 8B	f/2 8C	f/2 24A	f/2 24B	f/2 24C

11/21/22
13:00

Pellet cells by defrosting in ice and spinning @ 4°C and 10,000g for 10 min

14:00
Calculated SDS buffer volume:
    18 samples * 120 uL = 2,160 uL; will need to multiple 1,000 uL recipe x3
Made 3,000 uLSDS buffer:
    50 uL1M TEAB * 3 = 150 uL
    250 uL20% SDS * 3 = 750 uL
    2 uL1 M MgCl2 * 3 = 6 uL
    688 uLHPLC * 3 = 2,064 uL
    10 uL100X HALTprotease & phosphatase inhibitors * 3 = 30 uL
Vortexed

14:45
Sonicated each sample @ speed 3 for 20 sec then in ethanol + dry ice for 15 sec x3

15:55
Froze samples in -80

11/22/22
13:00
Calculated BCA reagent volumes
    200 uL * [(18 samples + 9 standards) * 3] = 16,200 uL total volume
    Round up to 17,000 for reagent A
    17,000 uL / 50 = 340 uLreagent B
Divided into two plates: one for spent, one for f/2. Each has triplicates of samples and standards.
    Made more BCA reagent solution for second plate's standards: 200 uL * (9 standards * 3) = 5,400 uL
    Round up to 6,000 for reagent A
    6,000 uL / 50 = 120 uLreagent B
Tested one well with 10 uL of sample + 200 uL reagent solution - Too purple! need to dilute 10:1
    Added 200 uL SDS Buffer + 20 uL sample to clean labelled 1.5 mL eppie tubes, vortexed

original green labels -> diluted red labels
original yellow labels -> diluted purple labels
200 uL * 18 = 3,600 uL -> rounded to 4,000 uL
Made 4,000 uLSDS buffer
50 uL1M TEAB * 4 = 200 uL
250 uL20% SDS * 4 = 1,000 uL
2 uL1 M MgCl2 * 4 = 8 uL
688 uLHPLC * 4 = 2,752 uL
10 uL100X HALTprotease & phosphatase inhibitors * 4 = 40 uL

14:06
Warmed plates @ 37°C for 30 min
14:36
Ran BCA and recorded outputs in Excel

BCA_Protein_112222_f2.xlsx

BCA_Protein_112222_spent.xlsx

11/29/22
Calculated how much protein lysate volume to aliquot for 50 ug protein
Graphed Standards vs Concentration to get scatter plot w/ line of best fit
Used equation from plot with highest R-sq value and solved for sample concentrations (x) by plugging in BCA output (y)
Averaged triplicates' concentrations (ug/mL) and divided by 1000 to convert to ug/uL
Divided 50 ug by sample concentration (ug/uL) to get volume needed
Subtracted volume from 40 uL to get volume of SDS buffer needed to have total volume of 40 uL
Pipetted undiluted sample and SDS buffer into new labelled 1.5 mL eppie tubes

S-traps 113022.xlsx

Sample	Average Concentration Undiluted (ug/uL)	Undiluted Protein lysate vol. needed for 50 ug S-Trap (uL): 50 / Conc.	SDS buffer needed for 40 uL total		Sample	Average Concentration Undiluted (ug/uL)	Undiluted Protein lysate vol. needed for 50 ug S-Trap (uL): 50 / Conc.	SDS buffer needed for 40 uL total
f/2 1A	2.086	23.97	16.03		s 1A	1.893	26.41	13.59
f/2 1B	2.154	23.22	16.78		s 1B	1.942	25.74	14.26
f/2 1C	2.059	24.28	15.72		s 1C	2.011	24.86	15.14
f/2 8A	2.000	25.00	15.00		s 8A	1.871	26.73	13.27
f/2 8B	1.791	27.92	12.08		s 8B	1.804	27.71	12.29
f/2 8C	2.052	24.37	15.63		s 8C	1.820	27.48	12.52
f/2 24A	2.161	23.14	16.86		s 24A	2.157	23.18	16.82
f/2 24B	2.172	23.02	16.98		s 24B	1.427	35.05	4.95
f/2 24C	1.782	28.06	11.94		s 24C	2.059	24.28	15.72

11/30/22

13:12

Thawed aliquoted samples

Calculated total volume 100 ug/uL enolase needed

8 uL 100 ug/uL enolase/sample * 18 samples = 152 uL 100 ug/uL enolase total

Diluted enolase

Made 280 uL 100 ug/uL enolase:

210 uLSDS buffer

70 uL400 ug/uL enolase

Vortexed

Added 8 uL 100 ug/uL enolase to samples

Added 0.5 uL 250 unit/uL benzonase, pipetting up and down to mix

Vortexed

13:34

Thawed 500 mM DTT and added 1.6 uL to each sample

Vortexed

13:41

Warmed @ 95°C for 10 min in blue heat block

Thawed 40 mM IAA

13:52

Added 3.3 uL 40 mM IAA

Vortexed

14:05

Incubated for 30 min @ room temp in drawer

14:35

Calculated volume needed of 12% aqueous phosphoric acid

40 uL + 8 uL + 0.5 uL + 1.6 uL + 3.3 uL = 53.4 uL

53.4 uL * 0.12 = 6.4 uL

14:43

Added 6.4 uL 12% aqueous phosphoric acid

Vortexed

Calculated volume needed of S-trap binding buffer

950 uL * 18 = 17.1 mL -> 20 mL recipe is OK

Made S-trap binding buffer with 2 mL1M TEAB + 18 mLMethanol

Added 350 uL S-trap binding buffer

Vortexed

15:09

Placed uncapped S-traps in clean labelled 1.5 mL eppie tubes

Added 136 uL sample to S-traps

Centrifuged @ 4000 g for 1 min then 6000 g for 1 min x2 to get all solution to pass through

Spun samples in 2 sets of 9, separating s and f/2 samples

Dumped flow through in waste falcon tube

Repeated 2x with rest of sample, had to bring up to 7500 g for 2 min

16:46

Washed 3x with 150 uL S-trap binding buffer, rotating 180° between washes

@ 7500 g for 2.5 min

Spun all 18 samples together

17:28

Made 50/50 chloroform/methanol solution in fume hood with 6 mL of each

Added 150 uL chloroform/methanol to S-traps

17:43

Centrifuged @ 4000 g for 1 min

Dumped flow through in MeOH/Chloroform waste in fume hood

Repeated 2x for 3 total washes

18:19

Washed 1x with 150 uL S-trap binding buffer

Moved S-traps to clean labelled 2 mL eppie tubes

18:49

Made 5 mL 50 mM TEAB

0.25 mL1M TEAB

4.75 mLHPLC water

Vortexed

Added 400 uL 50 mM TEAB each to 5 vials of 20 ug Trypsin to make 0.05 ug/uL Trypsin
Added 100 uL 0.05 ug/uL Trypsin to each S-trap
Loosely capped S-traps

19:07
Incubated @ 47°C for 1 hour

20:12
Centrifuged spent samples @ 4000 g for 30 sec to get rid of condensation
Added 80 uL 50 mM TEAB
20:15
Centrifuged spent samples @ 4000 g for 1 min
20:18
Added 80 uL 50% acetonitrile containing 0.2% formic acid to spent samples
20:23
Centrifuged f/2 samples @ 4000 g for 30 sec to get rid of condensation
Added 80 uL 50 mM TEAB
20:26
Centrifuged f/2 samples @ 4000 g for 1 min
20:30
Added 80 uL 50% acetonitrile containing 0.2% formic acid to f/2 samples
Froze elutions in -80

12/1/22
14:34
Dried down thawed elutions in speed vac
    Temp: cc, heat time: 2 hrs, vac pressure: 1
    Checked in after 30 min, then 25, then 20

15:53
Removed dried samples from speed vac and froze in -80

S-trap Protocol

🔗 S-trap Protocol.docx

Volume
Reagent
Time & Temp
Calculations

DAY 1 (~3 hours)

Make S-trap Solubilization/Lysis Buffer (SDS Buffer)

CALCULATE : Sample # * 120 uL = Total volume needed

For 1,000 uL:

- 50 uL 1M TEAB (cold room)
- 250 uL 20% SDS (bench)
- 2 uL 1 M MgCl2 (bench)
- 688 uL HPLC (above bench)
- 10 uL 100X HALT protease & phosphatase inhibitors (cold room, come in 100uL tubes)

Homogenization/Cell Lysis

1. Defrost samples on ice & immediately spin in large centrifuge @ 4°C and 10,000g for 10 min to pellet the cells
2. Sonication
    1. Add 120 uL SDS Buffer to each sample
    2. To cool: use ice bucket OR get small beaker and add approx. 20 uL ethanol (bench and next to sonicator), add dry ice pellets one at a time after each one is dissolved
    3. Sonicate at speed 3 for 20 sec then hold in ice for 30 sec OR cold ethanol for 15 sec, 3 times per sample
    4. Clean probe with ethanol in between samples
3. Can freeze in -80 or continue to BCA

BCA Assay

Determining protein concentration of your sample and ultimately how much protein you have. If you think you'll need a lot for s-traps consider diluting your sample.

If you think you have a very concentrated sample, do a quick dilution test with 4 wells: no dilution, 1:1, 1:5, and 1:10; incubate 30 min with reagent and chose the one that is lavender.

1. Create BCA standards according to manufacturer's instructions if not already made (store in -80)
2. CALCULATE 50:1 ratio A:B
    1. # wells you will use * 200 uL = Volume Reagent A (round up to whole mL)
    2. Volume Reagent A / 50 = Volume Reagent B
3. Aliquot Reagent A in a falcon tube, wait to add Reagent B (BCA box above bench)
4. Fill the plate
    1. When pipetting, press tip to the bottom of the well and only go down to first stop on pipette (make sure to be consistent and do this for all samples)
    2. Add 10 uL standards (A-I) along top 3 rows of the plate with replicates in columns
    3. Add 10 uL samples with replicates if you are doing them
5. Add Reagent B to Reagent A and vortex
6. Add 200 uL Reagent solution to every occupied well (do standards last) and gently shake, check for bubbles
7. Heat plate @ 37°C for 30 min (hallway)
8. Read plate using Varioskan
    1. Turn on machine then log into computer, click icon w/o box in upper left
    2. Click your folder then an old session, immediately save as with new name
    3. Adjust plate layout
    4. Insert plate into machine and run session, take picture of results!
    5. Save report to a USB
9. Fill out BCA Excel sheet to find sample protein concentrations in ug/mL and uL volume needed for 50 ug of protein
    1. Blank values (Standard I) should be around 0.15
10. Aliquot 50 ug protein for each sample in 1.5 mL tubes
11. Add SDS buffer so each sample has 40 uL total volume
12. Freeze in -80

DAY 2 (~5-6 hours)

Set heat blocks to correct temperatures you will need

Make SDS Buffer

For 1,000 uL:

- 50 uL 1M TEAB (cold room)
- 250 uL 20% SDS (bench)
- 2 uL 1 M MgCl2 (bench)
- 688 uL HPLC (above bench)
- 10 uL 100X HALT protease & phosphatase inhibitors (cold room, come in 20uL tubes)

Check pH: should be ~7.55

Dilution: make 100 ng/uL enolase

You want 8 uL 100 ng/uL enolase per 50 ug protein OR 0.16 uL 100 ng/uL enolase per 1 ug protein if you are working with <50 ug (change "8 uL" in equation if this is the case)

Thaw enolase on ice

CALCULATE : Sample # * 8 uL = Total volume 100 ng/uL enolase needed (uL)

For 140 uL:

- 35 uL 400 ug/uL enolase (-80 C4 rack in 35 uL aliquots)
- 105 uL SDS buffer

Prepare Samples For S-traps

1. Thaw samples
    - Check pH: should be ~7
    - Modify by adding 1M HCl if too basic or 1M TEAB if too acidic
2. Add 8 uL 100 ng/uL enolase
    - add less if low protein content
3. Add 0.5 uL 250 unit/uL benzonase (R4 freezer, green box), pipette up and down
    - Vortex
4. Heat @ 95°C for 10 min on blue heat block with thermometer (hallway) then cool for 5 min to unfold proteins
5. Spin in bench top centrifuge @ 4,000g for 30 sec to get rid of condensate on top of tubes
6. Add 500 mM DTT to final concentration 20 mM (R2-D2 freezer, thaw room temp) to break down disulfide bonds
    - CALCULATE: 500 mM * x = 20 mM (current vol. + x) where x = volume DTT
    - Add 500 mM DTT
    - Vortex
7. Heat @ 60°C for 10 min on then cool for 5 min

8. Spin in bench top centrifuge @ 4,000g for 30 sec to get rid of condensate on top of tubes
9. Add 500 mM IAA to final concentration 40 mM (R2-D2 freezer, thaw room temp in dark) to help trypsin access cleavage sites later on
    - CALCULATE: 500 mM * x = 40 mM (current vol. + x) where x = volume IAA
    - Add 500 mM IAA
    - Vortex
10. Incubate for 30 min in the dark at room temperature

Make S-trap Binding Buffer

- CALCULATE : Sample # * 950 uL = Total volume needed (mL)
- For 20 mL:
- 2 mL 1M TEAB (cold room)
- 18 mL Methanol (bench)
- Check pH: should be ~7.1

Make 50mM TEAB

- For 5 mL:
- Add 0.25 mL 1M TEAB (cold room)
- Add 4.75 mL HPLC water (bench)
- Keep on ice

11. Add 12% aqueous phosphoric acid (bench) at a 1:10 ratio (yields con. of approx. 1.2%) to denature proteins
    - CALCULATE : Current sample volume * 0.12 = Volume 12% aqueous phosphoric acid
    - Vortex - Do not centrifuge
    - Check pH: should be <2
12. Add 350 uL S-trap binding buffer
    - Vortex

S-traps

1. Label clean 2.0 mL eppie for each sample and set uncapped (save caps for later) S-trap inside, make a mark on rim
2. Add 136 uL sample to S-traps
3. Spin in bench top centrifuge @ 4,000g for 1 min
    - Dump flow through in waste

4. Repeat steps 2-4 until all sample has gone through, rotating S-traps 180° between washes
5. Wash 3x with 150 uL S-trap binding buffer, rotating S-traps 180° between washes
    - Dump flow-through in waste

50/50 Methanol Chloroform Extraction

1. CALCULATE : Sample # * 450 uL = Total volume needed (mL) (round up to nearest whole number)
2. Divide total volume in half to get volume of both methanol and chloroform needed for 1:1 ratio
3. Add methanol and chloroform to a falcon tube (same volumes) (bottom right cabinet under fume hood)
4. Add 150 uL chloroform/methanol mixture to S-traps
5. Spin in bench top centrifuge @ 4,000g for 1 min
    - Dump flow through in waste (bottom right cabinet under fume hood) in fume hood
6. Repeat steps 4&5 2x for a total of 3 washes, rotating S-traps 180° between washes
7. Wash 1x with 150 uL S-trap binding buffer, dumping flow through
8. Move S-trap column to a clean labeled 1.5 mL eppie (**PEPTIDE CONTAINING TUBE**)

Digestion

1. Make 0.05 ug/uL Trypsin
    - CALCULATE : Sample # * 100 uL = Total volume
    - Add 2000 uL 50 mM TEAB to each 100 ug Trypsin vial (R4 freezer) OR 400 uL 50 mM TEAB to each 20 ug Trypsin vial (R4 freezer)
    - Keep on ice
2. Add 100 uL 0.05 ug/uL Trypsin to column, make sure there is no bubble!
3. Loosely cap the S-trap columns
4. Incubate @ 47°C for 1 hour
5. Spin in bench top centrifuge @ 4,000g for 30 sec to get rid of condensate on top of tubes
6. Add 80 uL 50 mM TEAB
7. Spin in bench top centrifuge @ 4,000g for 1 min to elute

8. Add 80 uL 50% acetonitrile containing 0.2% formic acid (bench) to elute hydrophobic peptides
9. Spin in bench top centrifuge @ 4,000g for 1 min to elute
10. Dispose of column, all peptides should now be in your tube
11. Freeze in -80

DAY 3 (~2 hours)

Dry Down

1. Thaw samples
2. Speed Vac samples
    1. Turn on in order 1 -> 2 -> 3, waiting few min for indicator on 1 to light before turning on other two
    2. Lift lid at sides and evenly space out samples
    3. Temp is cc, heat time is 2 hrs, vac pressure is 1
    4. Select Manual run for the time to count up
    5. Wait for click to make sure there are no issues
    6. Remove when liquid is evaporated
3. Resuspend in 50 uL 2% acetonitrile containing 0.1% formic acid

SP3 Analysis 11/28/22

Using diluted f/2 24hr A, B, C
P. piscicida

1. 200 uL starting vol of diluted protein mixture
2. Add 18.4 uL TCEP (drawer) + 11.6 HPLC and mix at room temp, 700 rpm, 30-60 min
3. Thaw IAA (50 uL aliquot top R2D2 freezer) in drawer
4. Thaw DTT (top R2D2 freezer) on ice
5. Mix approx. 80 mg AmBic (above centrifuge) + 10 mL HPLC, put on ice
6. Dilute IAA 1:1 with 50 uL HPLC and add 15 uL to sample + 5 uL HPLC, let sit in drawer for 30 min
7. Add 5.5 uL DTT + 19.5 uL HPLC to sample, let sit for 10 min

8. Add small scoop of beads
9. Add 700 uL ACN
10. Let sit for -- min then set on magnet stand and pipette out liquid
11. Make 80% Ethanol using 40 mL Ethanol + 10 mL HPLC
12. Add 1 mL to beads let sit 1 min, put back on magnet stand
13. Carefully pipette out liquid, can let air-dry for couple min to let Ethanol evaporate - do 12 & 13 2x
14. Make AmBic wash
    - 87 uL AmBic stock (step 5)
    - 5 uL Enolase
    - 5 uL PRTC (PRTC + 40uL AmBic stock)
    - 3 uL Trypsin (20 ug Trypsin + 40uL AmBic stock)
15. Let sit for few min
16. Carefully pipette out liquid into clean labelled eppie, this contains peptides
17. Speed vac