# **SUPPLEMENTARY DOCUMENTATION S1**

**The Galaxy** Instance used for our metaproteomics gateway can be accessed by using a web-based user interface accessed by the URL "<u>z.umn.edu/metaproteomicsgateway</u>". The Tool Pane (left side column) contains a list of available software tools in the Galaxy instance. The central portion of the interface is called the Main Viewing Pane, where the users can set operating parameters for the tools, edit and view workflows comprised of multiple tools, and also view results for and from data analyses. The right-side column of the interface is the History Pane, which shows the active History.



Firstly, in order to use the Galaxy instance, register as a user and create login/password credentials.

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Protein/Peptide Search Algorithms	must be at least three characters in length and contain only lower-case letters, numbers, dots, underscores, and dashes (",		
FASTA manipulation			
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Workflows			
All workflows			

## Using a Galaxy tool: Database generation

The first step in the analysis is to import the required input data files, which users can download from the Shared Data Libraries. Once imported, these data files will appear on the History pane. [click on Shared Data tab, and then click on "Data Libraries".



In the list of shared data, click on "Metaproteomics Training". Click "Metagenome\_sixgill.fastq", select the file, and click on "Import to History". This folder contains one file in the fastq format, which consists of the biological sequence and its corresponding quality scores data.

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**Sixgill** (Six-frame Genome-Inferred Libraries for LC-MS/MS) is a tool for using shotgun metagenomics sequencing reads to construct databases of **'metapeptides'**: short protein fragments for database search of LC-MS/MS metaproteomics data. The main Sixgill command is **sixgill\_build** (http://noble.gs.washington.edu/proj/metapeptide/), which builds a metapeptide

Once the file is imported to your history, click on ""Shared Data -> Workflows" and then on "Metagenomics to Metaproteomics for training". Click on import workflow.

Name	
Metaproteomics training workflow	•
Metagenomics to Metaproteomics	- training 🔻
	Import
H1_ABRF2017 SW1 Metagenomics	Survey File

The workflow will be imported to your personal workflow library. Click on the "Workflow" tab and select the imported workflow. A drop-down menu appears, click on "Run". The workflow will appear on the main viewing pane. Click on "Run workflow" which will select the input files and build a FASTA database.



This is one way to perform Database generation. You could also use a publicly available database or use the Protein Database Downloader tool within Galaxy to download protein FASTA databases from single organism as well as metaproteomic databases.

The **Protein Database Downloader** tool helps in downloading a FASTA file of specified protein sequences for comparison with experimental MS/MS data in search algorithm. The Protein Database downloader can download sequences from Uniprot, cRAP, EBI Metagenomics, HOMD (Human Oral Microbiome Database) and Human Microbiome Project body sites (airways, blood, gastro-intestinal tract, oral, skin and urogenital tract). You can also download it through Custom-URL.

# Using a Galaxy Workflow:

Now that the protein database ready, we will use the metaproteomics workflow on the MGF file inputs for database searching, taxonomy analysis and functional analysis. Firstly, the input files **Mascot generated format** (MGF) files need to be imported. Please note, that these are trimmed MGF files from Bering Strait dataset along with a trimmed Sixgill-generated metapeptide FASTA file (*J. Proteome Res.*, 2016, *15* (8), pp 2697–2705). For functional analysis, we will also need a Gene Ontology mapping file (http://geneontology.org/ontology/go-basic.obo).

To obtain these files, click on "Shared Data" and select "Data Libraries". Click on "Metaproteomics training". Select all the files in the folder (excluding the Metagenome Sixgill.fastq) and import it to history. Name the history as 'Metaproteomics Training Output'. Once imported your history should have 3 MGF files, 1 Gene Ontology File and 1 metapeptide FASTA database. In order to prepare all the MGF fractions for database search (and subsequent steps) so that it generates a single output, we will need to create a Dataset collection. Select all the MGF files in your input history and create a Dataset collection.



The Dataset collection can be labeled as "BeringStrait MGFs". By creating a dataset collection, all the MGF files can be searched together to generate a single search output.

Now that you have inputs for running a workflow, we can import the Metaproteomics training workflow. For this, select "Shared Data -> Workflows" and click on "Metaproteomics training workflow" to import the workflow.

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Metaproteomics training	g workflow 👻
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To run the workflow, go to the **"Workflow" tab** and select the recently imported workflow. When you run the workflow, it will appear on your Main viewing pane. Ensure that you see appropriately labeled input files in the boxes and click on **Run**.

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Once you click **Run Workflow**, all the tools mentioned in the workflow will queue up in the history panel. These tools will be grey while in queue, yellow while running and green in color once the task has finished. Once completed, the history pane on right would look like the one shown below:

History	2 ♥ □
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11.94 MB	299
20: GO Terms: Cellular Localization	• / ×
19: GO Terms: Molecular Functions	• / ×
18: GO Terms: Biological Processes	• / ×
17: Genera   PSMs   Peptides	• / ×
16: sqlite db of data 13 and data 9	• / ×
15: Peptides and PSMs	• / ×
14: sqlite db of data 9, data 11, and data 5	• / ×
13: Unipept pept2lca on data 10 tsv	• / ×
12: UniPept Phylogenetic Tree	• / ×
11: Unipept pept2prot on data 10 tsv	• / ×
10: query results on data 9	• / ×
9: Peptide Shaker on data 7: PSM Report	• / ×
8: Peptide Shaker on data 7: Parameters	• / ×
7: SearchGUI Results	• / ×
6: BeringStrait_MGFs a list of 3 datasets	×
5: Gene_Ontology_Terms.tabular	• / ×
4: FASTA_Bering_Strait_Trimmed_metapeptid es_cRAP.fasta	• / ×
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## Workflow component: Spectral Matching

In the generated History, Steps **7-9** are related to database searching, where we use SearchGUI and PeptideShaker to search the MGF files against the Metapeptide Database.

**SearchGUI** (Proteomics, 2011, 11:996-999), bundles several open-source and freely available sequence database searching programs, facilitating analysis of MS/MS data using more than one algorithm and increasing confidence in results. SearchGUI has been deployed in Galaxy. Here we will use it to match MS/MS spectra to sequences in our FASTA database.

**PeptideShaker** (Nature Biotechnol. 2015, 33(1):22–24), runs multiple search engines (X! Tandem, OMSSA, MS-GF+ and others) on any number of MGF peaklists using the SearchGUI application and combines the results.

SearchGUI performs protein identification using various search engine, in this workflow for simplicity, we will only use one database search engine i.e. X!Tandem.

#### Parameters used for SearchGUI:

Protein digestion parameters: Trypsin, with 2 maximum missed cleavages The precursor ion tolerance is 10 ppm, with fragment tolerance of 0.02 Da Minimum/maximum charge of ions: 2/6 Fragment ions searched: b and y Fixed protein modification: Carbamidomethylation of C Variable protein modification: Oxidation of M

PeptideShaker processes the output file from SearchGUI. It infers proteins from matched peptide sequences and applies statistical metrics to assign confidence to identified peptides and proteins. Within this workflow, the "Default" options are selected, with relevant parameters as follows:

#### Parameters used for PeptideShaker:

The maximum FDR value (%) at protein level is 1.0, peptide level is 1.0 and PSM level is 1.0 Minimum and maximum peptide length are 6 and 30 respectively Maximum precursor error is 10.0 ppm Outputs selected: PSM report (tabular) and Certificate of Analysis (text).

For this workflow, the PSM report was selected along with the Certificate of Analysis. The History contains the PSM report generated for our workflow.

# Workflow component: Data Processing

Step **10** utilizes the Query Tabular tool that was used to select those distinct PSMs that had a confidence of more than 95%.

## Parameters for Query Tabular:

SELECT distinct sequence FROM psm WHERE confidence >= 95 ORDER BY sequence

The tabular output that contains distinct microbial peptides that is then subjected to UniPept Analysis (*Proteomics* 2015, **15**, 1437–1442).

Unipept (<u>http://unipept.ugent.be/</u>) is an open-source web application developed at Ghent University that is designed for metaproteomics data analysis with a focus on interactive taxonomic data visualizations. Unipept is powered by an index containing all Uniprot Entries, NCBI taxonomy and custom lowest common ancestor (LCA). This helps in performing biodiversity analysis of large and complex metaproteome samples. It's available in API and command line interface. Unipept also has tools for selecting unique peptides for targeted proteomics for comparing genomes based on peptide similarity.

In this workflow, Unipept (Steps **11-13**) is used for both taxonomic and functional analysis. Detected peptides were given taxonomic assignments by Unipept version 2.0.1. For all tryptic peptides with no missed cleavages present in UniProtKB, Unipept assigns a lowest common ancestor (LCA) taxon from the NCBI Taxonomy Database, the most- granular taxon common to all organisms containing the peptide. For peptides with missed tryptic cleavages, Unipept calculates an LCA based on the LCAs associated with all completely cleaved peptide sequences contained in the peptide.

## Workflow component: Taxonomy analysis

**Taxonomy analysis** (Step **12-13**) uses the Unipept pept2lca function to generate the taxonomic lowest common ancestor for each peptide. Unipept analysis using "pept2lca" function generates two outputs: a. The JavaScript Object Notation (JSON) output, which will be used for visualization.

b. The tabular ( .tsv) output

# Parameter used for Unipept Taxonomy Analysis: Unipept application: pept2lca: lowest common ancestor Equate isoleucine and leucine: YES (isoleucine (I) and leucine (L) are equated when matching tryptic peptides to UniProt records) retrieve extra information: NO (Return the complete lineage of the taxonomic lowest common ancestor, and include ID fields.) names: YES return the names in complete taxonomic lineage

The JSON file (**Step 12**) opens a Unipept tree viewer in which user can interactively explore the taxonomy tree represented in our sample.





Figure 1: Unipept taxonomy tree

The "Query tabular" and "SQLite to tabular" tools produce a table to generate number of PSMs for each peptide (Steps 14 - 15) so that a follow up experiment can be performed, if needed.

#### Parameters for PSMs corresponding to Peptides

SELECT sequence as "peptide", count(id) as "PSMs" FROM bering psms WHERE confidence >= 95 GROUP BY sequence **ORDER BY sequence** 



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Steps 16 and 17 are the "SQLite to tabular" and "Query tabular" tools to produce a table for each Gene Ontology category summarizing the number of peptides and PSMs associated with each Gene Ontology description.

#### Parameter for Query Tabular:

SELECT lca.genus,count(psm.sequence) as "PSMs",count(distinct psm.sequence) as "DISTINCT PEPTIDES" FROM psm LEFT JOIN lca ON psm.sequence = lca.peptide WHERE confidence >= 95 GROUP BY lca.genus ORDER BY PSMs desc, 'DISTINCT PEPTIDES' desc

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## Workflow component: Functional analysis

Steps **11**, **14**, **18** - **20** describe the tools used for functional analysis. For Functional analysis, the detected peptide sequences are converted to protein identifications using the Unipept Pept2Pro module.

Parameter used for Unipept Functional Analysis:

Unipept application: pept2prot: UniProt entries containing a given tryptic peptide

## Equate isoleucine and leucine: YES

(isoleucine (I) and leucine (L) are equated when matching tryptic peptides to UniProt records)

## retrieve extra information: YES

(Return additional information fields: taxon\_name, ec\_references, go\_references, refseq\_ids, refdeq\_protein\_ids, insdc\_ids, insdc\_protein\_ids.

Later, the Query Tabular tool is used to generate, "biological process", "molecular function", and "cellular compartment" GO term outputs along with associated PSMs. For this, the Unipept Pep2Pro output, PSM report and Gene Ontology term (GO) (http://geneontology.org/ontology/go-basic.obo) category annotations are used.

# Gene Ontology Categories

## a. Biological Processes:

All the peptides and PSMs that matched to a particular Biological Process are listed in the tabular form. **Biological Processes** within Gene Ontology Categories is based on the series or collection of molecular functions.

*For example: Translation:* encompasses multiple steps that are involved during the process of translation.

# Parameters used for Determining Biological Processes:

SELECT go.description, count(distinct bering\_psms.sequence) as "bering\_peptides", count(distinct bering\_psms.id) as "bering\_psms" FROM go JOIN bering\_prot\_go ON go.go\_id = bering\_prot\_go.go\_reference JOIN bering\_prot on bering\_prot\_go.id = bering\_prot.id JOIN bering\_psms ON bering\_prot.peptide = bering\_psms.sequence WHERE go.aspect = 'biological\_process' GROUP BY go.description ORDER BY bering\_peptides desc,bering\_psms desc

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Fetch Alignments	ATP synthesis coupled proto	n transport						3		<u>es</u>	
Statistics	DNA repair							3		145 lines, 1 comments	
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for peptides	Fc-epsilon receptor signaling	g pathway						1			
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#### b. Molecular Functions:

In the SQLite to tabular tool, we select all the peptides and PSMs that matched to a particular **Molecular Function** to be listed in the tabular form. **Molecular Functions** category is based on the functions of a gene product.

For example: **transport activity** is the molecular function of the peptide as its function is to transport molecules.

#### Parameters used for Determining Molecular Function:

SELECT g.description, count (distinct b.peptide) as "bering\_peptides", count (distinct b.id) as "bering\_psms" FROM go as g JOIN (SELECT go.description, bering.peptide, bering\_psms.id FROM go LEFT OUTER JOIN bering ON go.go\_id = bering.go\_reference JOIN bering\_psms ON bering.peptide = bering\_psms.sequence GROUP BY go.description, bering.peptide, bering\_psms.id) as b ON g.description = b.description WHERE g.aspect = 'molecular\_function' GROUP BY g.description ORDER BY bering\_peptides desc,bering\_psms desc



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Fetch Alignments	RNA binding							5	20	format: tabular, da	atabase: ?		
Statistics	tRNA binding							5	19	BOCW?			
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FASTA manipulation	translation elongation fac	tor activity						5	25	1 #deccription			honi
MetaProteomics	DNA-directed 5'-3' RNA	olymerase activ	ty					5	19	ATP binding			32
MS Data Conversion	formate dehydrogenase (	NAD+) activity						4	25	structural consti	tuent of ri	bosome a	29
Sequence Database Tools	4 iron, 4 sulfur cluster bit	nding						4	17	DNA binding		2	23
Protein/Peptide Search	heme binding						3	3	21	rRNA binding		1	19
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#### c. <u>Cellular Localization:</u>

In the SQLite to tabular tool, we select all the peptides and PSMs that matched to a particular **Cellular Localization** to be listed in the tabular form. **Cellular Localization** category is based on the location at the levels of subcellular structures and macromolecular complexes.

#### For example: **Cytoplasm**

# Parameters used for Determining Cellular Localization: SELECT g.description, count(distinct b.peptide) as "bering\_peptides", count(distinct b.id) as "bering\_psms" FROM go as g JOIN (SELECT go.description, bering.peptide, bering\_psms.id FROM go LEFT OUTER JOIN bering ON go.go\_id = bering.go\_reference JOIN bering\_psms ON bering.peptide = bering\_psms.sequence GROUP BY go.description, bering.peptide, bering\_psms.id) as b ON g.description = b.description WHERE g.aspect = 'cellular\_component' GROUP BY g.description ORDER BY bering\_peptides desc,bering\_psms desc



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Get Data	translation	30	148	Metaproteomics Training Ou	itput
Send Data	protein refolding	16	63	20 shown	
Collection Operations	protein folding	12	53	11.94 MB	8 .
Text Manipulation	regulation of transcription, DNA-templated	11	146	Process Contract	
Filter and Sort	transmembrane transport	9	55	20: GO Terms: Cellular Local	• / ×
Join, Subtract and Group	carbohydrate transport	7	69	ization	
Convert Formats	transcription, DNA-templated	7	22	43 lines, 1 comments	
Extract Features	chromosome condensation	6	59	ioimat. tabuiai, database. <u>r</u>	
Fetch Sequences	chaperone-mediated protein folding	4	8	<b>В Ө С ш ?</b>	۰
Fetch Alignments	ATP synthesis coupled proton transport	3	20	1	
Statistics	DNA repair	3	12	#description	
Graph/Display Data	regulation of translation	3	11	cytoplasm	
FASTA manipulation	rRNA processing	2	11	ribosome	
MotaProteomics	amino acid transport	2	6	outer membrane-bounded perip	lasmic space
MC Data Companies	RNA processing	2	5	cell outer membrane	_
MS Data Conversion	mRNA catabolic process	2	s		
Sequence Database Tools	Entrer-Doudoroff nathway through 6-phosphonluconate	1	13	19: GO Terms: Molecular Fu	• / ×
Protein/Peptide Search	DNA damage response detection of DNA damage	1	9	incuons	
Algorithms	DNA damage response, detection of biot damage	1	9	18: GO Terms: Biological Pr	• # X
for peptides	Ec-ensilon recentor signaling nathway	1	9	ocesses	
Deprecated Tools	C2/M transition of mitotic coll curle	1	9	17: Genera   PSMs   Peptides	@ / X
Deprecated Tools	L-kanna® kinase /NE-kanna® signaling	1	9		
Workflows	NK coccade	1	9	16: sqlite db of data 13 and	● / ×
All workflows	MARK cascade	1	9	data 9	
	MARK cascade	1	9	15: Peptides and PSMs	@ / X
	MyD86-dependent toll like reseater signaling pathway		9		(0.01-0.100)
	MyDoo-independent toll-like receptor signaling pathway	1	9	14: sqlite db of data 9, data	• / ×
	Nik/Nr=kappab signaling	1	9	11, and data 5	
	Noten signamig patiway	1	9	13: Unipept pept2lca on dat	● / ×
	T cell essenter signalize pathway	1	9	<u>a 10 tsv</u>	
	The decenter signaling pathway	1	9	12: UniPent Phylogenetic Tr	
	The separate of MARY and the	1	9	ee	
	activation of MAPK activity	1	9		
	adipose tissue development	1	9	11: Unipept pept2prot on da	• / ×
	anaphase-promoting complex-dependent catabolic process	1	9		
	autopnagy	1	9	10: query results on data 9	● # ×
	central protein metabolic process	1	9	O Dentido Shaker en data 7	
	central protein mouncation process	1	9	9. reptide snaker on data 7: PSM Report	• / ×
	circadian rhythm	1	9	1 Stor In Sector	
	determination of adult lifespan	1	9	8: Peptide Shaker on data 7:	• / ×
	endosomai transport	1	9	Parameters	
	energy homeostasis	1	9	7: SearchGUI Results	
<	error-tree transiesion synthesis	1	9	10	>

In summary, this single Galaxy workflow takes in MGF input files and searches it against a metaproteomic database to generate PSM report. The PSM report is later used to parse out microbial peptides that are used for taxonomy analysis and functional analysis using Unipept.