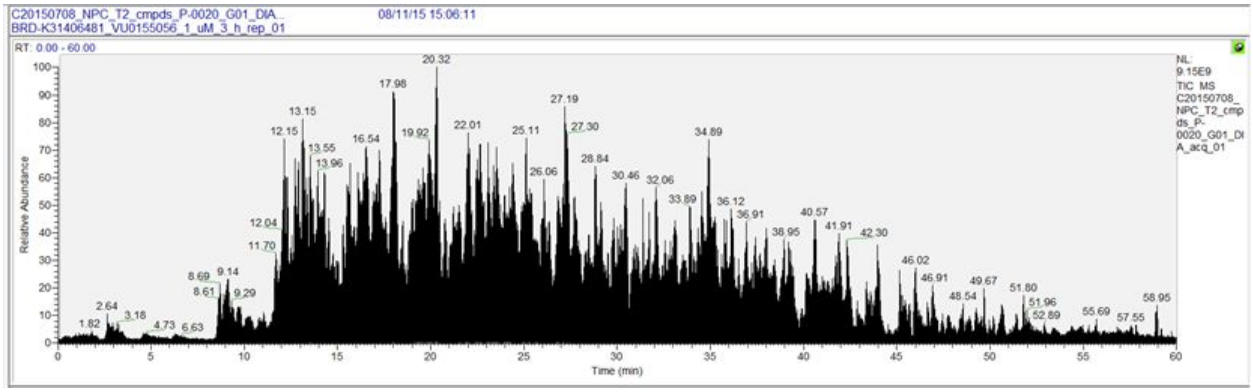
P100 Data Acquisition Guidelines

The purpose of this data sheet is to give general guidelines and tips for acquiring DIA data for P100. This is not an exhaustive database of everything that can go wrong, just the basics.

DIA data acquisition

On a newly conditioned column with the instrument running well you can expect the TIC of a P100 DIA run to look like the one below. The file size is expected to be between 2.5-3.5Gb.

TIC intensity ideally high e9's, does shape look normal? Keep an eye out for sparse MS2 spectra, TIC only reflects the MS1, file size low could be bad MS2's



Over the course of running the plate, the overall file size will decline somewhat due to column aging. **This is normal.** You should be worried if the file sizes start to drop drastically and continues across multiple sets of replicates. Sometimes a drug treatment will kill the cells and result in very little signal for its triplicates but this isn't indicative of a problem unless multiple triplicate sets have low signal.

If the file size drops below **1Gb**, you should check the MS quality by running Jurkats and will likely need to change the column. This can also be indicative of the MS being dirty and it may need to be cleaned.

A good rule to monitor how the column and MS are doing is to run Jurkats once per quadrant and search them as soon as they're finished running. In general, a good Jurkat peptide count number for Copernicus is between ~34,000 and 37,000, but good DIA data can still be acquired with peptide counts as low as ~28,000. Use good judgment.

Summary**:**
Ideal File Size: **1.5-3.5Gb**
Inject Volume: **3uL**

Current DIA method:
C:\\Xcalibur\methods\LINCS\CurrentDIA\P100_DIA_11amu_Overlap_22amuwindow_60min_50msIT_400-1000_27loopcount

DDA data acquisition

Data dependent acquisitions of P100 samples are needed for:

- The eventual creation of spectral libraries
- Phosphopeptide Enrichment Quality Metrics

DDA runs should be done at the beginning of running a plate and back to back with the sample's DIA run (DIA followed by DDA). Historically, we've collected DDA runs for 2uL injections of B01, G01, A12, H12, and D06 in order to assess if there are plate effects if an enrichment fails.

The TIC should look like the same as the DIA run (the method uses the same gradient) and the file size is expected to be approximately 0.5Gb.

Summary:
Ideal File Size: **0.5Gb**
Inject Volume: **2uL**
Current DDA method:
C:\\Xcalibur\methods\LINCS\LINCS_60min_DDA_2uL_centroid_USEforSAMPLES

Searching DDA data using SpectrumMill

Browse to \\musketeer\msdataSM\LINCS
Create a new folder for the current P100 LINCS plate
Create subfolders named B01, G01, A12, H12, and D06
Place copies of the appropriate DDA files into their respective folders

Using internet explorer, open SpectrumMill server **dunlop**:
http://dunlop/millhome.htm

Open 'Workflows' and scroll to 'Jenn\QE_PhosphoProteome_Human'

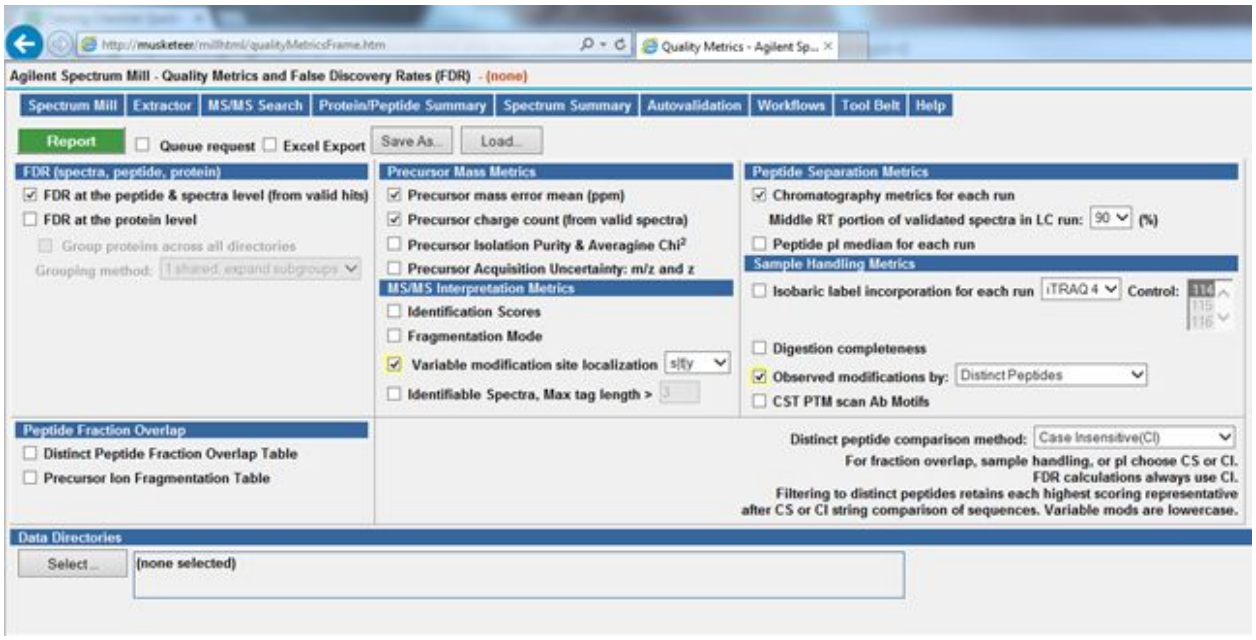
- The parameters used in this workflow can be found below

Select the directories that you just created on musketeer and press 'Execute'
SpectrumMill will search the data according to the parameters specified. This should take 1-2 hours for 5 files.

In SpectrumMill, return to the home page and browse to 'Quality Metrics and FDR'
Select the data directories that you just searched
Check the following parameters to return relevant quality metrics:

- FDR at the peptide & spectra level (from valid hits)

Precursor mass error mean (ppm)
Precursor charge count (from valid spectra)
Chromatography metrics for each run, Middle RT portion of the validated spectra in LC run: 90%
Observed modifications by: Distinct Peptides
Distinct peptide comparison method: Case Sensitive
Variable modification site localization: s|t|y



Press ‘Report’ to return quality metrics

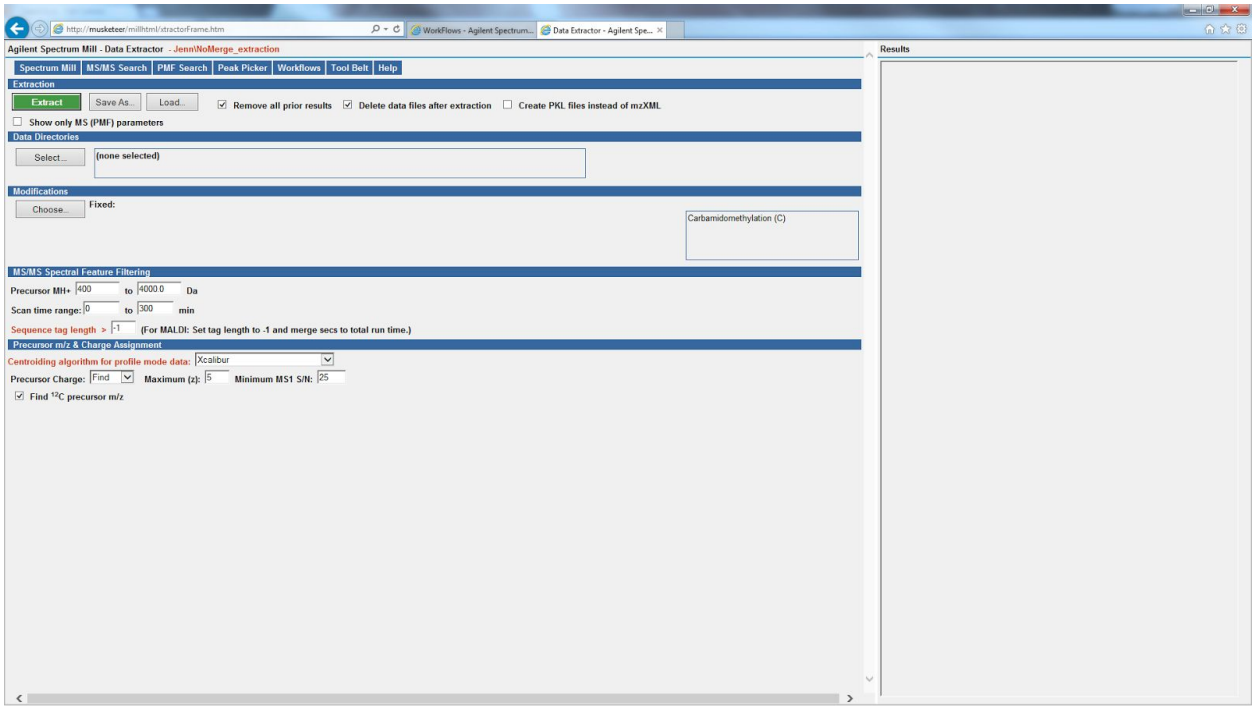
For a quick look at how well the phosphopeptide enrichment performed, look at the following metrics:

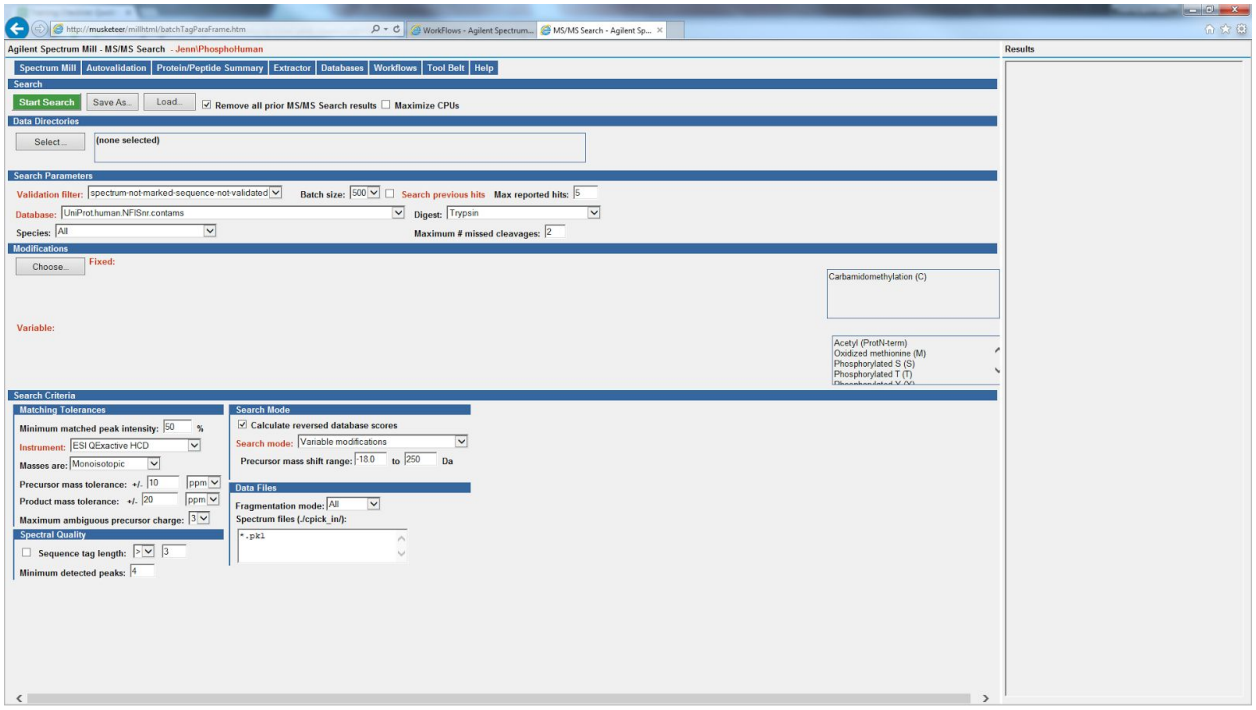
s|t|y Sites spectra (%): Ideal is above 90%, average result is 85% but >70% is usually acceptable
s|t|y Sites spectra (#): Average result is around 8,000
Distinct Peps CI Total (#): Average result is around 5,000 to 7,000 peptides identified

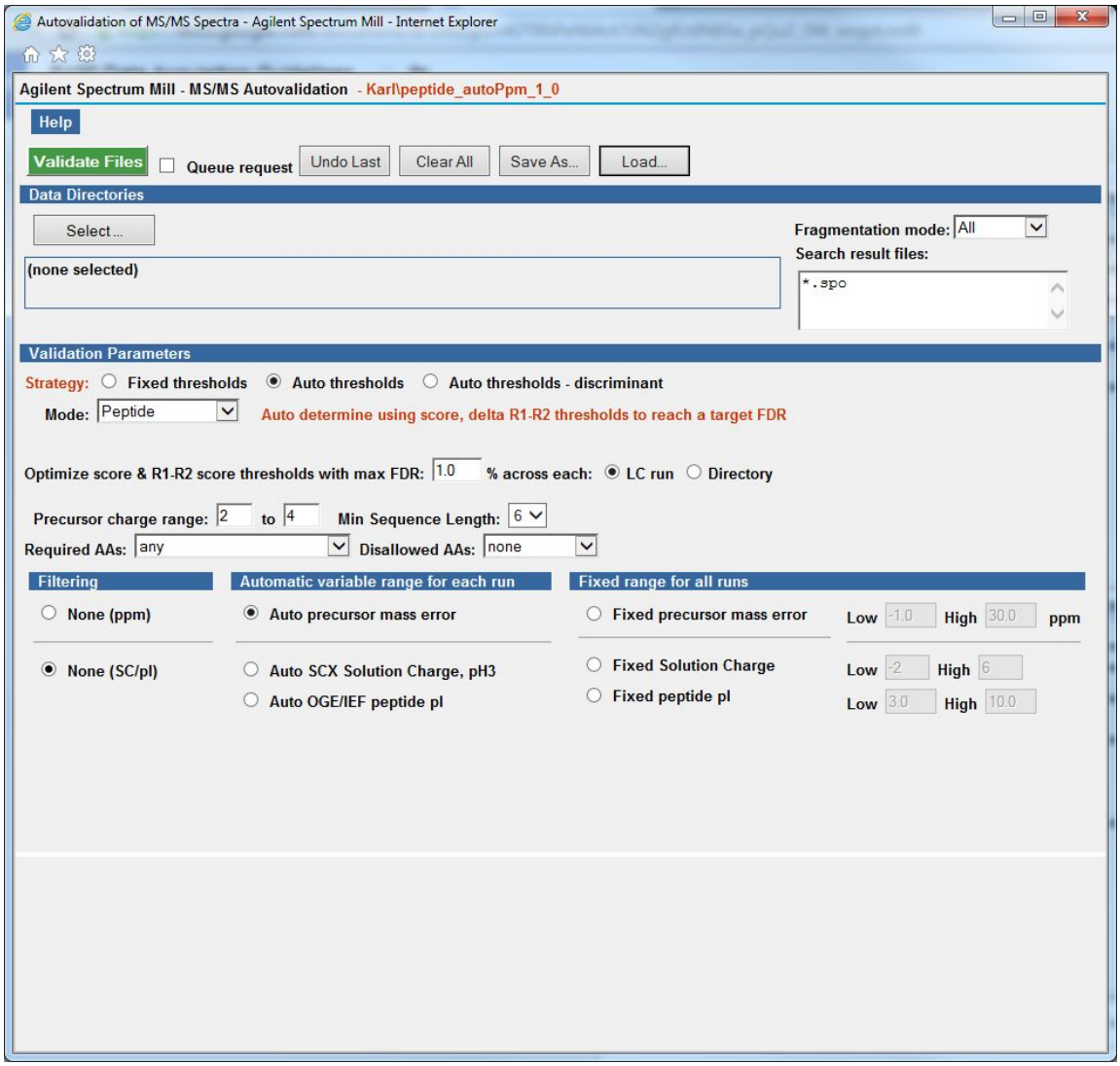
Another way to assess enrichment efficacy is to make a SpectrumMill report of phosphopeptide only intensities and another with intensities of all peptides ID’d in the run. The percentage of total intensity is generally >95% phosphopeptides. Briefly, to do this:

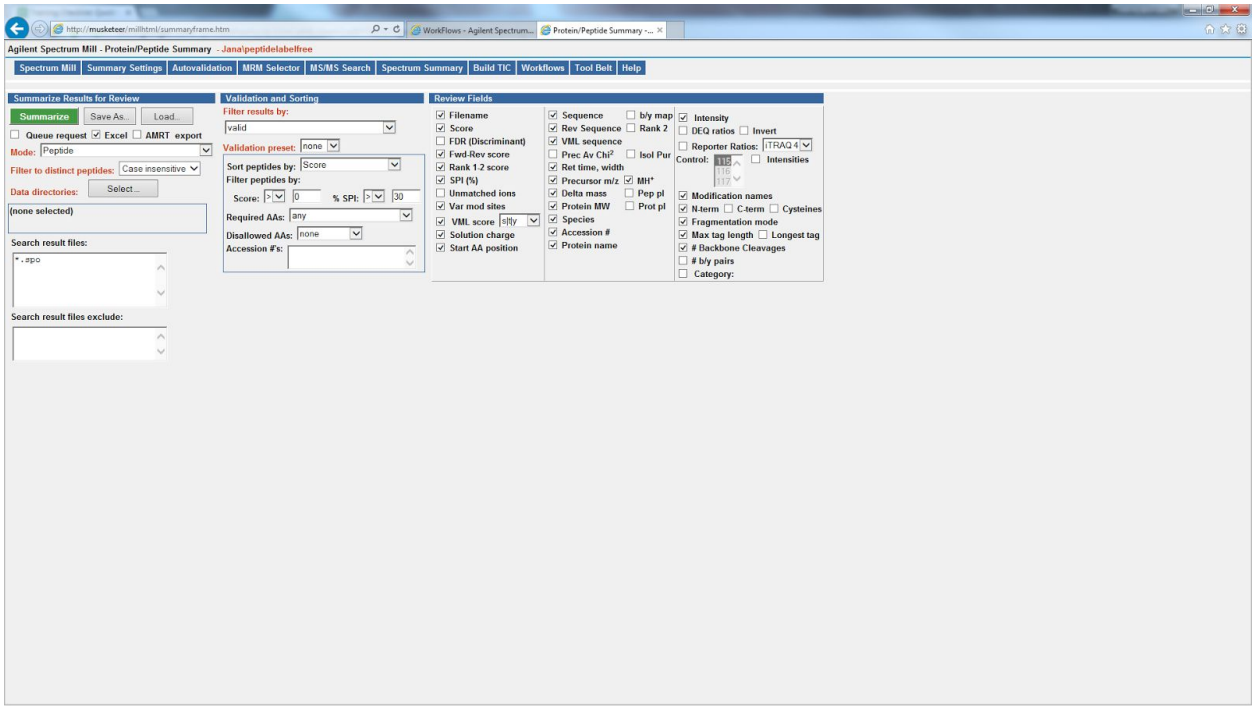
Create a SM peptide report of all peptides ID’d
Create a SM peptide report of only s|t|y peptides ID’d
Sum the intensity of both reports
Divide phosphopeptide intensity by total intensity to get % phosphopeptides

DDA Search Workflow Parameters: IN THIS ORDER






Autovalidation of MS/MS Spectra - Agilent Spectrum Mill - Internet Explorer
Agilent Spectrum Mill - MS/MS Autovalidation - Karl\peptide_autoPpm_1_0
Help
Validate Files
Queue request
Undo Last
Clear All
Save As...
Load...
Data Directories
Select ...
(none selected)
Fragmentation mode: All
Search result files:
*.spo
Validation Parameters
Strategy: Fixed thresholds Auto thresholds Auto thresholds - discriminant
Mode: Peptide
Auto determine using score, delta R1-R2 thresholds to reach a target FDR
Optimize score & R1-R2 score thresholds with max FDR: 1.0 % across each: LC run Directory
Precursor charge range: 2 to 4 Min Sequence Length: 6
Required AAs: any Disallowed AAs: none
Filtering
None (ppm)
None (SC/pI)
Automatic variable range for each run
Auto precursor mass error
Auto SCX Solution Charge, pH3
Auto OGE/IEF peptide pI
Fixed range for all runs
Fixed precursor mass error
Low -1.0 High 30.0 ppm
Fixed Solution Charge
Low -2 High 6
Fixed peptide pI
Low 3.0 High 10.0


http://musketeer/millhtml/summaryframe.htm
WorkFlows - Agilent Spectrum...
Protein/Peptide Summary -...
Agilent Spectrum Mill - Protein/Peptide Summary - Jana\peptidelabelfree
Spectrum Mill
Summary Settings
Autovalidation
MRM Selector
MS/MS Search
Spectrum Summary
Build TIC
Workflows
Tool Belt
Help
Summarize Results for Review
Summarize
Save As...
Load...
Queue request
Excel
AMRT export
Mode:
Peptide
Filter to distinct peptides:
Case insensitive
Data directories:
Select...
(none selected)
Search result files:
*.apo
Search result files exclude:
Validation and Sorting
Filter results by:
valid
Validation preset:
none
Sort peptides by:
Score
Filter peptides by:
Score:
% SPI:
Required AAs:
any
Disallowed AAs:
none
Accession #'s:
Review Fields
Filename
Score
FDR (Discriminant)
Fwd-Rev score
Rank 1-2 score
SPI (%)
Unmatched ions
Var mod sites
VML score
Solution charge
Start AA position
Sequence
Rev Sequence
VML sequence
Prec Av Chi²
Ret time, width
Precursor m/z
Delta mass
Protein MW
Species
Accession #
Protein name
b/y map
Rank 2
Isol Pur
MH+
Pep pI
Prot pI
Intensity
DEQ ratios
Invert
Reporter Ratios:
iTRAQ 4
Control:
Intensities
Modification names
N-term
C-term
Cysteines
Fragmentation mode
Max tag length
Longest tag
Backbone Cleavages
b/y pairs
Category: