

Discovery to Targets for a Phosphoproteomic Signature Assay: One-stop shopping in Skyline

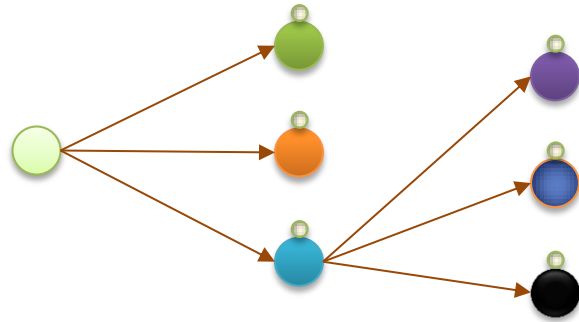
Jake Jaffe

Skyline Users Meeting

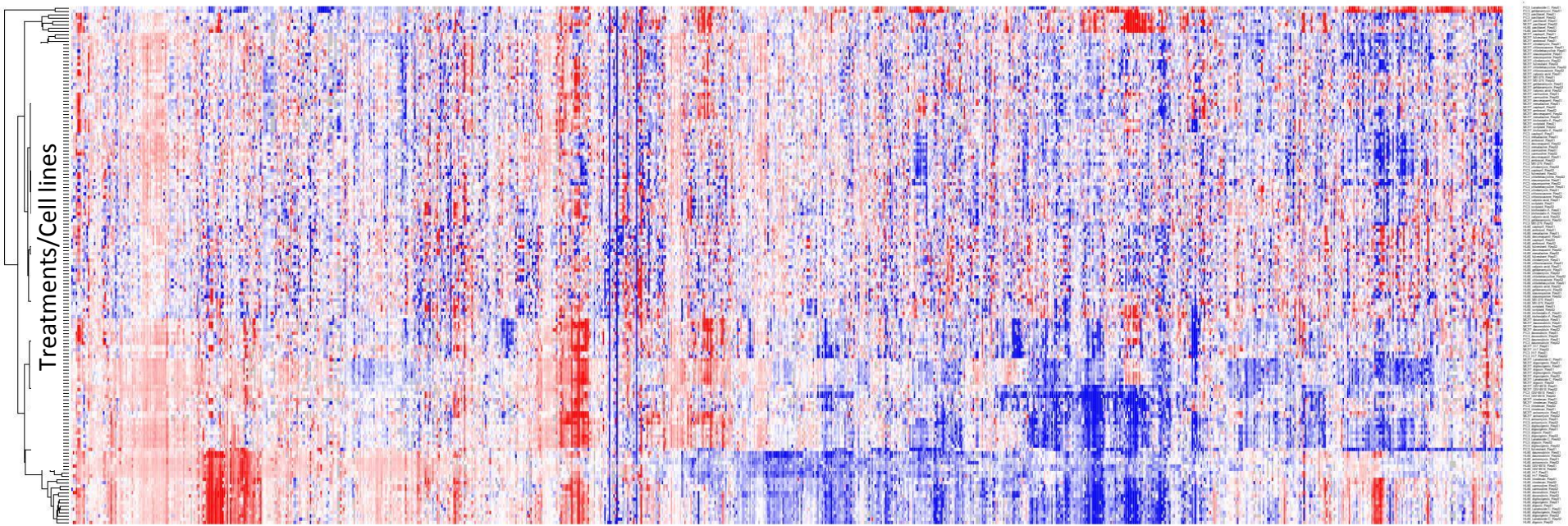
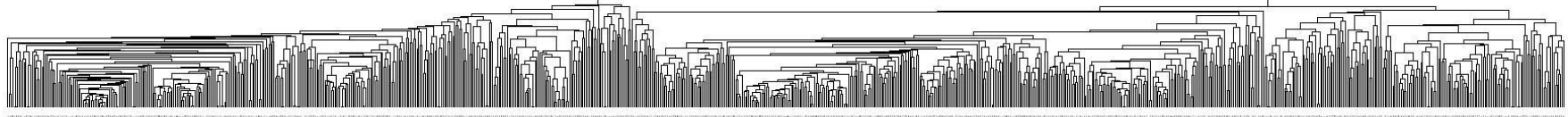
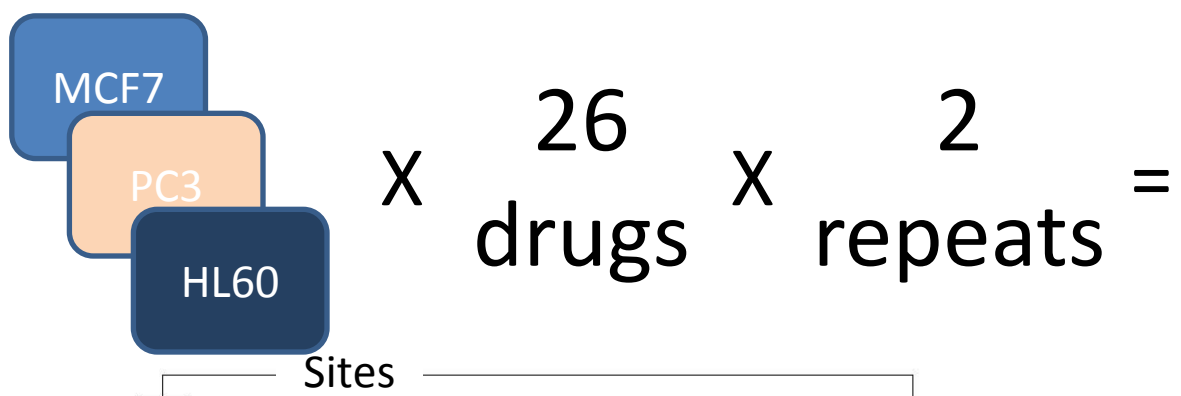
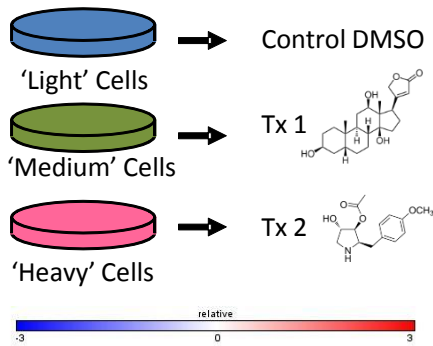
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Idea: Phosphosignaling is coordinated!

- Phosphosignaling is likely coordinated
 - Kinase-substrate relationships tend to be 1-to-many
 - *A priori* expectation of coordinate regulation of sites



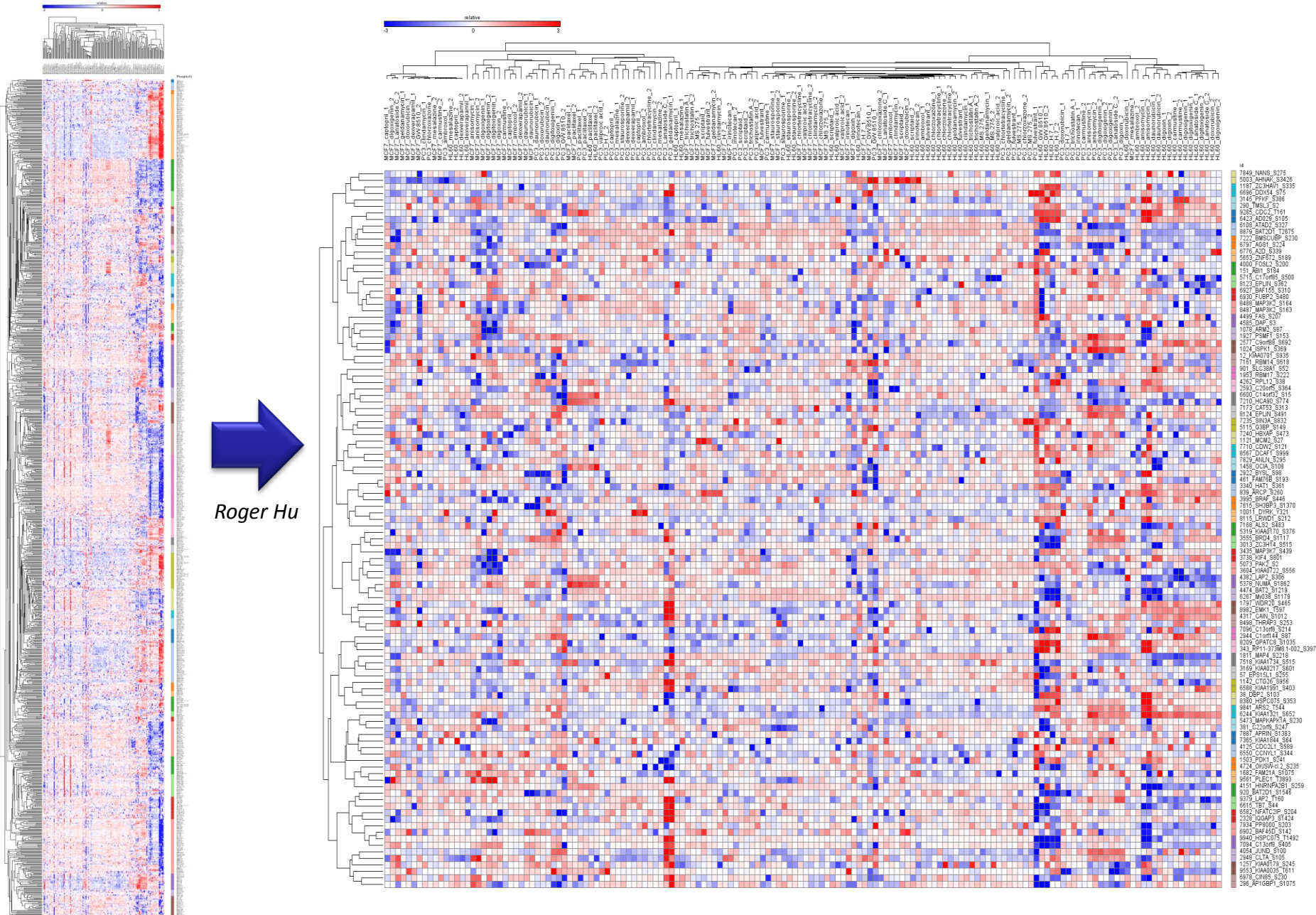
- Don't need to monitor every phosphosite
- Can we identify a few that we can reliably monitor?
 - Go ***wide*** instead of ***deep***



- Largest systematic set of perturbations with phosphoproteomics readout in existence
 - Over 10,000 phosphosites observed
- Over 1,200 sites present in >75% of all experiments

Jinal Patel, Xiaodong Lu

Dimensionality reduction: >1000 to 55 with 2x probe redundancy



Now for the hard part...
Building the P100 Assay

Optimal Phosphosite Quant Requires Multiple Inputs

- Level of phosphopeptide
 - Picked “best” representative peptides from each cluster
 - Observability, site localization, etc.
- Level of non-phosphorylated cognate
 - Picked based on above
- Level of a non-involved peptide
 - Control for protein level
 - Gleaned from all in-house MaxQuant data

Assay Optimization Strategy

- ✓ Identify and order peptides

- Configure Skyline document
 - Sequence identities
 - Spectra libraries

- Characterize peptides
 - QC + establish RT properties

- Refine spectral libraries

- Configure final assay

Step 1: Get Sequences into Skyline

The screenshot displays the Skyline software interface. On the left, the 'Peptide List' table contains the following data:

Peptide Sequence	Protein Name
HMFVHYH	MAP3K2_CL11
DRS[+80]SPPPGYIPDELHQVAR	MAP3K2_CL11
DRSSPPPGYIPDELHQVAR	MAP3K2_CL11
LGMLSPEGTC[+57]K	FAS_CL12
IPHLAIHLQR	DAP_CL12
LGMLS[+80]PEGTC[+57]K	FAS_CL12
SS[+80]PPEGKLETK	DAP_CL12
SSPPEGKLETK	DAP_CL12
LGM[+16]LS[+80]PEGTC[+57]K	FAS_CL12
DGAWGAFR	FAS_CL12
LGM[+16]LSPEGTC[+57]K	FAS_CL12
EDLESSGLQR	ARM2_CL13
LEAAEER	LAP18_CL13
RNS[+80]SEASSGDFLDLK	ARM2_CL13
RNSSEASSGDFLDLK	ARM2_CL13
RAS[+80]GQAFELILS[+80]PR	LAP18_CL13
RASGQAFELILSPR	LAP18_CL13

The 'Peptide Settings' dialog box is open, showing the 'Modifications' tab. The 'Structural modifications' section includes:

- Dimethyl (R)
- Phospho
- Oxidation (M)
- Phospho (ST)
- Phospho (Y)
- ArgAsymmetricDimethyl

The 'Max variable mods' is set to 3 and 'Max neutral losses' is set to 1. The 'Isotope label type' is set to 'heavy'. The 'Isotope modifications' section includes:

- Label:13C(6)15N(4) (C-term F)
- Label:13C(5) (V)
- Label:13C(6) (C-term K)
- Label:13C(6) (C-term R)
- Label:13C(6)15N(2) (C-term K)
- Label:2H(4) (K)

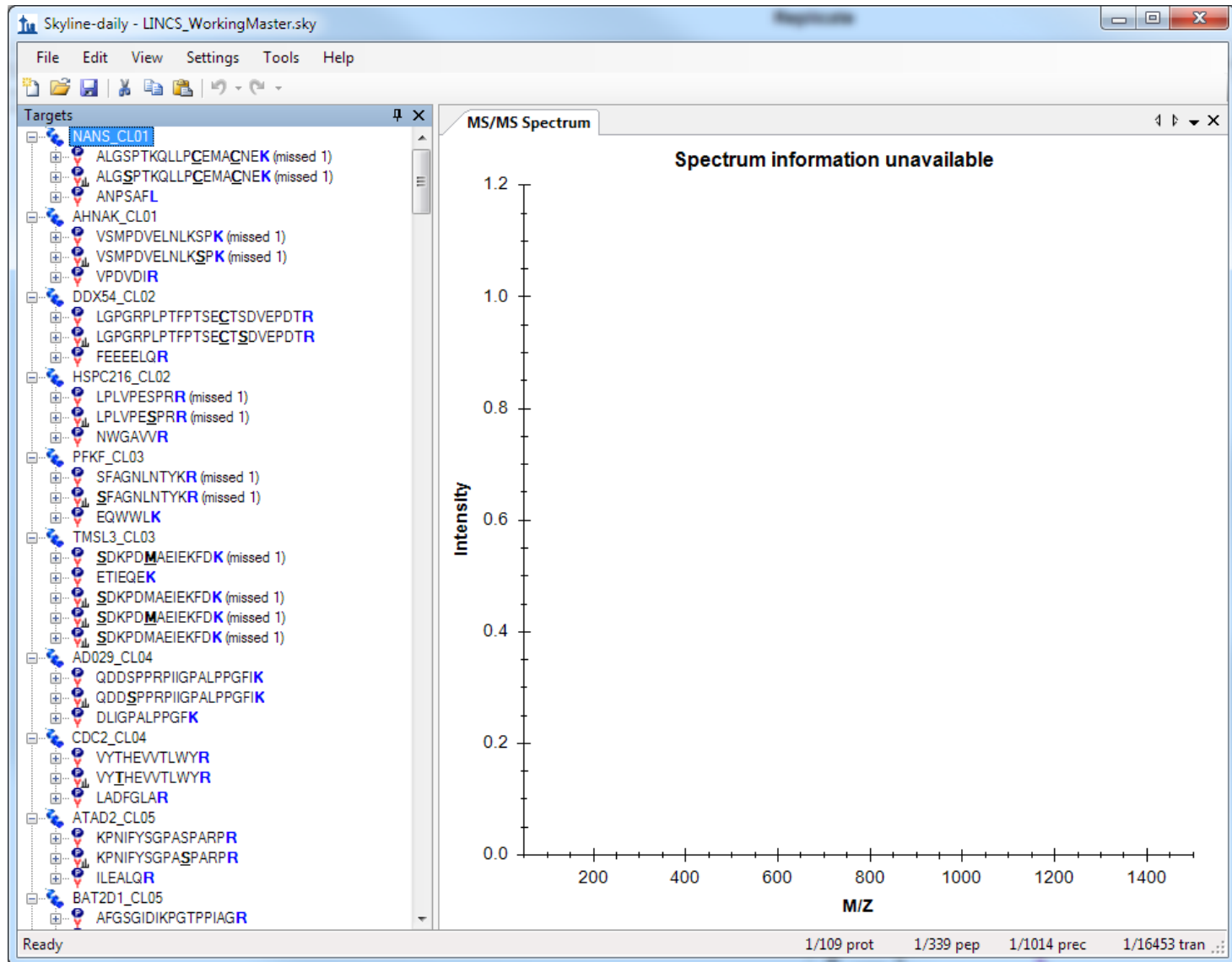
The 'Internal standard types' section includes:

- light
- heavy
- medium

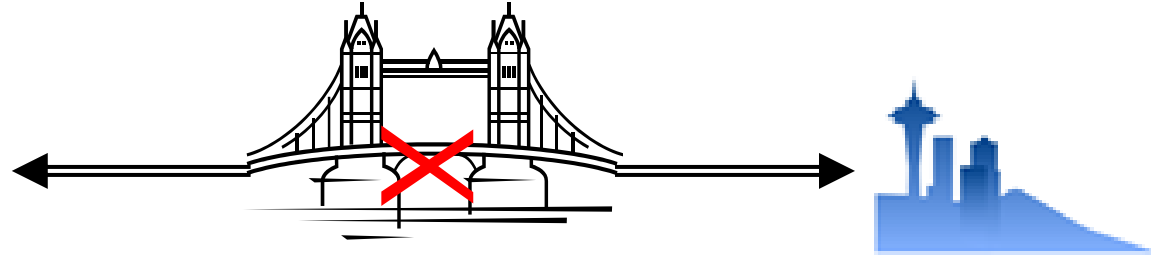
Buttons for 'OK' and 'Cancel' are visible at the bottom of the dialog box. At the bottom of the main window, there are buttons for 'Check for Errors', 'Insert', and 'Cancel'.

- Peptide shorthand makes it easy to insert modified sequences
 - Make sure modifications are present in document!

Entire assay document configured in one fell swoop



Discovery and Targeted Platforms are Different



■ MaxQuant

- Used for all discovery data processing
- Performed all SILAC quantification
- MEGA result file
 - MS/MS table (txt)
 - 2.8 GB

■ Skyline

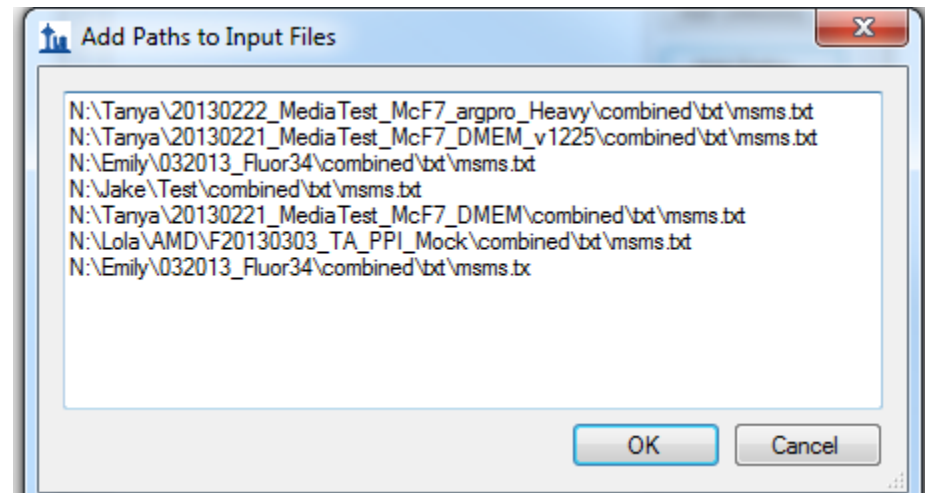
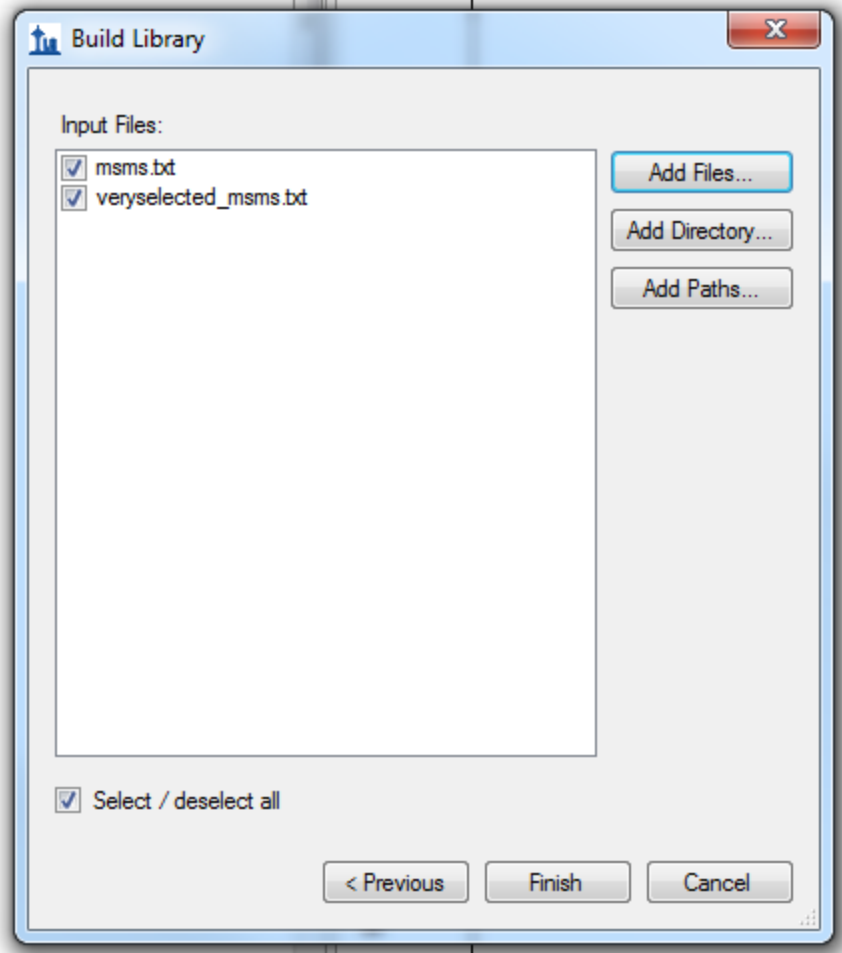
- Targeted proteomics environment of choice
- ***Now supports MaxQuant / Andromeda formats***

Skyline Spectral Library Building

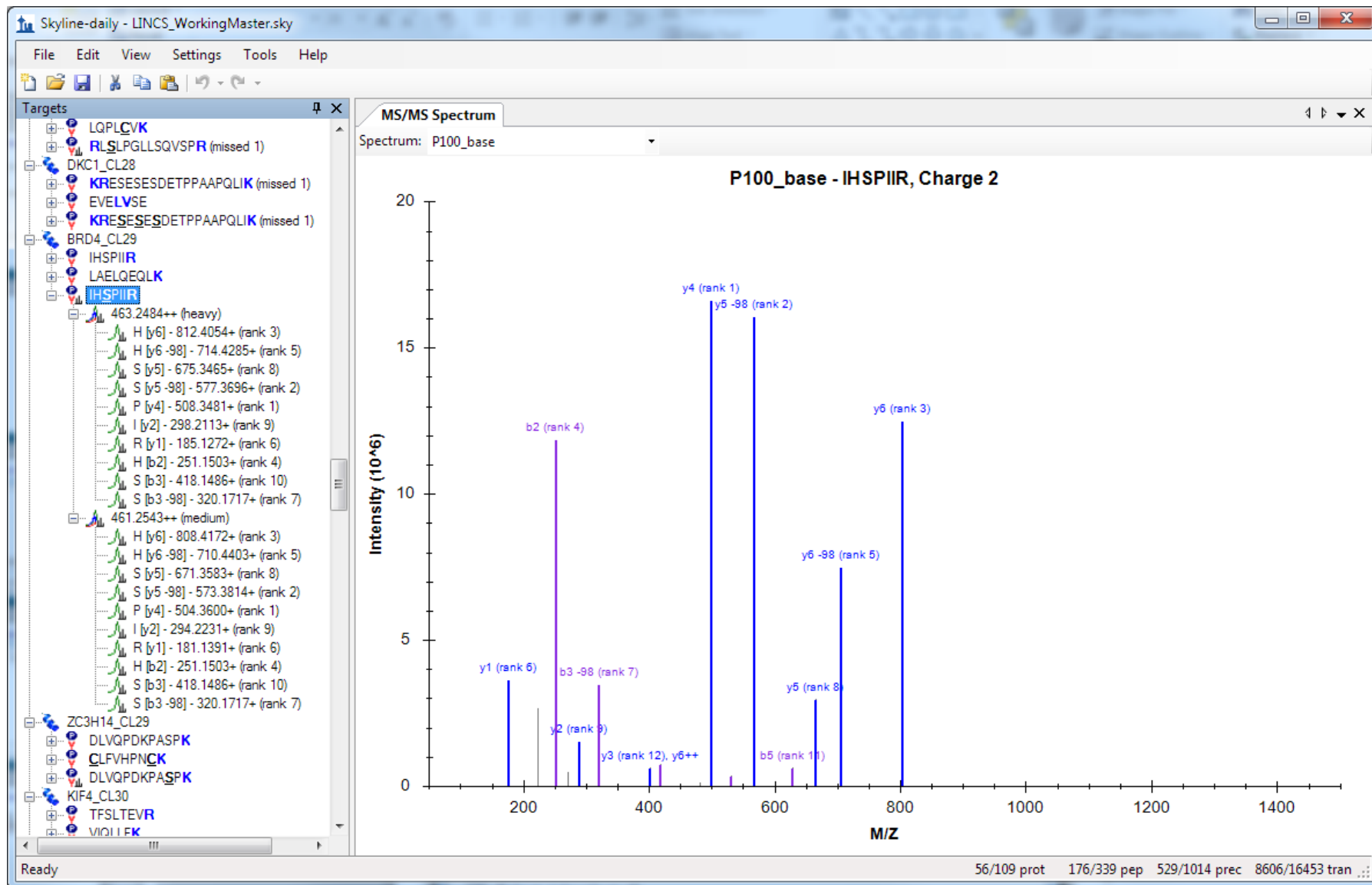
The screenshot displays the Skyline software interface for building a spectral library. The main window shows a list of targets on the left and an MS/MS spectrum on the right. Two dialog boxes are overlaid on the interface:

- Build Library Dialog:**
 - Name: MyNewLib
 - Output Path: C:\temp\sky\LocalCopies\lib\MyNewLib.blib
 - Action: Create
 - Keep redundant library:
 - Cut-off score: 0.01
 - Filter for document peptides: (circled in red)
 - Lab Authority: broadinstitute.org
 - Library ID: MyNewLib
- Peptide Settings Dialog:**
 - Libraries: MQ1225test, P100BaseLocal, MQ1305test, HistoneApr2012, Rigged, TestP100
 - Pick peptides matching: Library
 - Rank peptides by: (dropdown menu)
 - Limit peptides per protein:

Easily done even when data are distributed



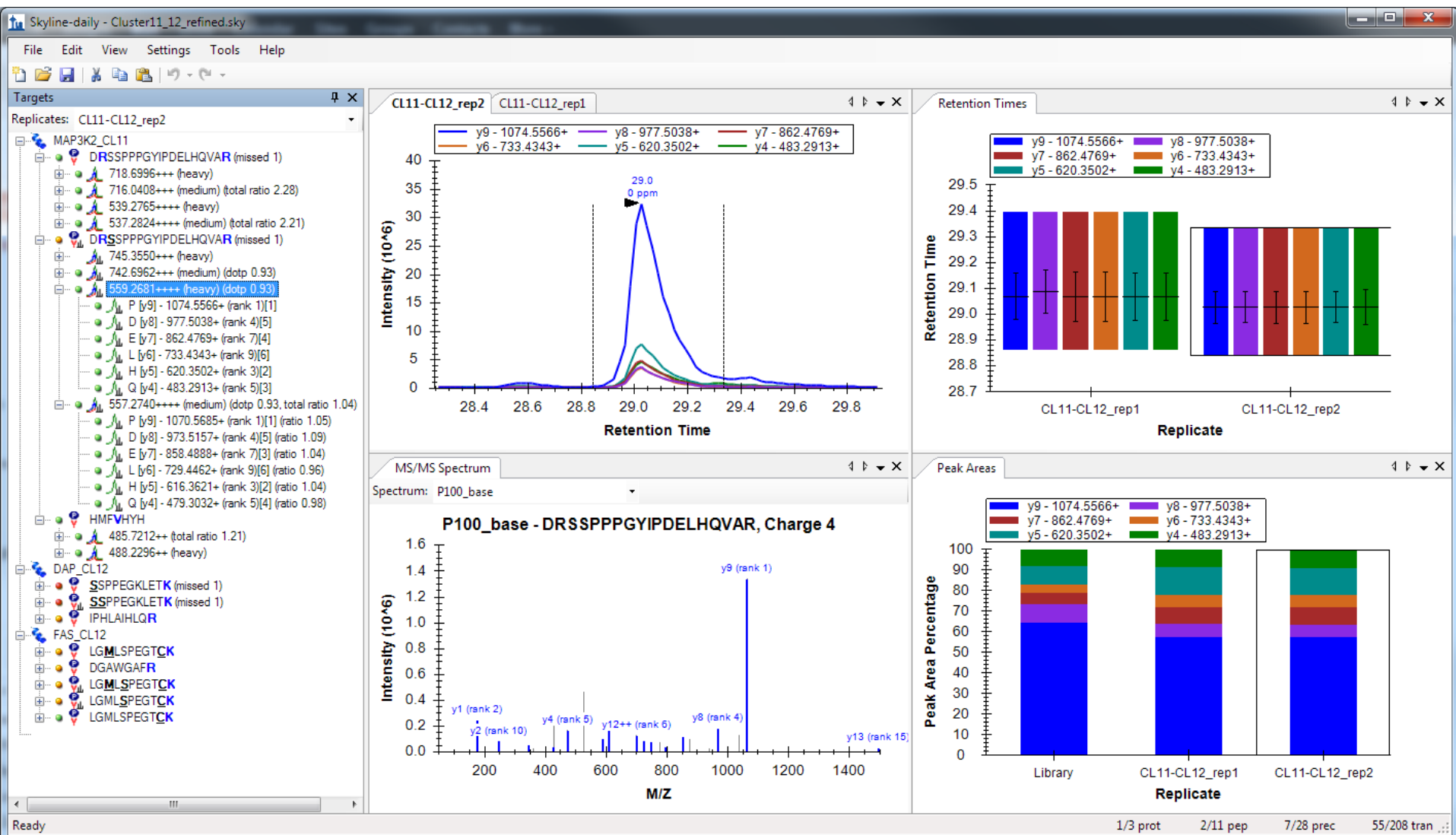
Instant Focused Spectral Library from > 2.8Gb Raw Spectra



Peptide QC and RT Establishment

- Run mixtures of peptides conforming to analysis clusters
 - QExactive MS
 - Inclusion lists pulled from Skyline doc
 - Unscheduled...but fully targeted
- Full-scan hi-res MS enables proof positive ID
 - Allows comparison to library as a whole spectrum
 - Mass accuracy increases confidence
 - Can observe potential interferents when porting to triple quad

Full-scan QC allows for proof-positive refinement



Assay Optimization Strategy Revisited

- ✓ Identify and order peptides

- ✓ Configure Skyline document
 - Sequence identities
 - Spectra libraries

- ✓ Characterize peptides
 - QC + establish RT properties
 - Will later use iRT strategy for assay scheduling

- ✓ Refine spectral libraries

- Configure final assay

Distribution of the assay with Panorama



- All of our work will be available to other labs
 - To implement the assay at multiple sites

- Ensure data analysis consistency

- Clearing house of assay data

- Communication with other project data infrastructures

Conclusions

- Skyline has contributed immensely to rapid configuration of our assay:
 - Easy sequence import – even for modified peptides
 - Easy spectral library creation based on discovery data
 - Helpful in validation of synthetic peptides
 - Sets groundwork for scheduling targeted assays
 - Makes assay portable
 - Panorama extension will make assay distributable
- Feedback / support cycle of Skyline development can drive projects in real time

Acknowledgements

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