Preparation

View → text zoom → large

Set properties text size to 14 point

CONTEXT

- We are going to use Skyline to generate the isolation list to be used for our DIA method

Transition Settings – Full Scan

Click this last

Transition Settings

Prediction | Filter | Library | Instrument | Full-Scan

MS1 filtering

Isotope peaks included: Count

Precursor mass analyzer: Orbitrap

Peaks: 3

Resolving power: 35,000 At: 200 m/z

Isotope labeling enrichment: Default

MS/MS filtering

Acquisition method: DIA

Product mass analyzer: Orbitrap

Isolation scheme:

Resolving power: 17,500 At: 200 m/z

Retention time filtering

- (•) Use only scans within 2 minutes of MS/MS IDs
- ( ) Use only scans within 5 minutes of predicted RT
- ( ) Include all matching scans

OK Cancel

Edit isolation scheme

Edit Isolation Scheme

Name:

20mzDIA

OK

Cancel

Use results data isolation targets

Isolation width: 2 m/z

Deconvolution:

Asymmetric

Prespecified isolation windows

Calculate...

Graph...

Start	End

Deconvolution: None

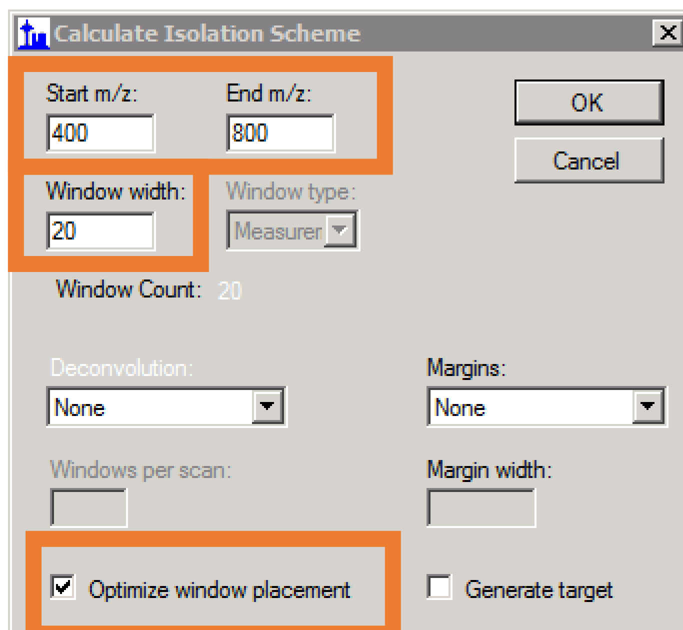
Margins: None

Windows per scan:

Specify target

Calculate isolation scheme

400 – 800 because the sample data covers this, normally do 500 - 900



Graph isolation scheme

Edit Isolation Scheme

Name: 20mzDIA

OK

Cancel

Use results data isolation targets

Isolation width: 2 m/z

Deconvolution:

Asymmetric

Prespecified isolation windows

Calculate...

Graph...

Start	End
400.4319	420.4410
420.4410	440.4501
440.4501	460.4592
460.4592	480.4683
480.4683	500.4774
500.4774	520.4865
520.4865	540.4956
540.4956	560.5047
560.5047	580.5138
580.5138	600.5229
600.5229	620.5319
620.5319	640.5410

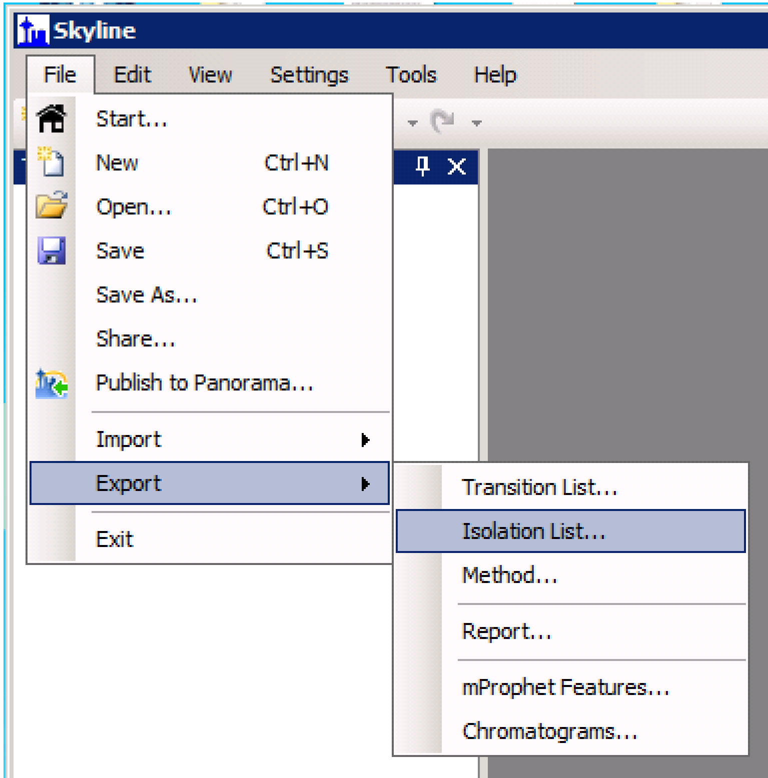
Deconvolution: None

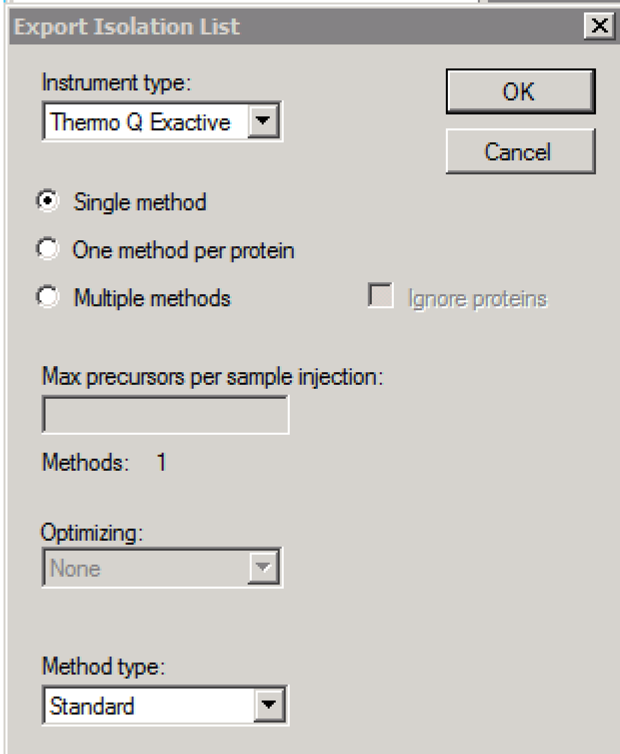
Margins: None

Windows per scan:

Specify target

Export Isolation List





methods / DIAIsolationList.csv

Save Skyline doc to skydocs / DIA.sky and open QE method editor (Xcalibur → Instrument Setup)

Full MS Settings

DIA.meth - Thermo Xcalibur Instrument Setup

File Help

Q Exactive - Orbitrap MS

Waters nanoACQUITY

Global Lists

Lock Masses | Inclusion | Exclusion | Neutral Loss | Tag Masses

Tune Files

External Hardware

Chromatogram

Scan Groups

Full MS – SIM

DIA

0 10 20 30 40 50 60 70 80

time (min)

Experiments

General

Full MS – SIM

AIF

Full MS / AIF

Full MS / dd-MS² (TopN)

Targeted-SIM

Targeted-MS²

Targeted-SIM / dd-MS²

Full MS / AIF / NL dd-MS²

DIA

Full MS

Properties

Properties of the method

Global Settings	
User Role	Standard
Use lock mass	best
Chrom. peak	15 s
Time	
Method durati	80.00 min

Properties of Full MS — SI

General	
Runtime	0 to 80 min
Polarity	positive
Full MS — SIM	
Resolution	35,000
AGC target	3e6
Maximum IT	55 ms
Scan range	495 to 905 m/z

Experiment Setup | Summary

Ready

DIA Settings

DIA.meth - Thermo Xcalibur Instrument Setup

File Help

Q Exactive - Orbitrap MS

Acquity

Waters nanoACQUITY

Global Lists

Lock Masses Inclusion Exclusion Neutral Loss Tag Masses

Tune Files

External Hardware

Chromatogram

Scan Groups

Full MS – SIM

DIA

0 10 20 30 40 50 60 70 80

time (min)

× 1.00

Experiments

General

- Full MS – SIM
- AIF
- Full MS / AIF
- Full MS / dd-MS² (TopN)
- Targeted-SIM
- Targeted-MS²
- Targeted-SIM / dd-MS²
- Full MS / AIF / NL dd-MS²
- DIA

Full MS

DIA

Properties

Properties of the method

Global Settings	
User Role	Standard
Use lock mass	best
Chrom. peak	15 s
Time	
Method durati	80.00 min

Properties of DIA

General	
Runtime	0 to 80 min
Polarity	positive
Default charge state	2
DIA	
Resolution	17,500
AGC target	1e6
Maximum IT	auto
Loop count	10
MSX count	1
MSX isochronous ITs	on
Isolation window	20.0 m/z
Fixed first mass	—
NCE / stepped NCE	26
Spectrum data type	Centroid

Import Isolation list

CONTEXT

- We are going to start defining our document – the set of peptides, and transitions we would like to extract. Informed by the DDA data.

Transition Settings → Filter

Transition Settings

Prediction | Filter | Library | Instrument | Full-Scan

Precursor charges: 2, 3

Ion charges: 1

Ion types: y, b, p

Product ions

From: ion 3

To: last ion - 1

Special ions:

- [x] N-terminal to Proline
- [ ] C-terminal to Glu or Asp
- [ ] iTRAQ-114
- [ ] iTRAQ-115
- [ ] iTRAQ-116
- [ ] iTRAQ-117
- [ ] TMT-126
- [ ] TMT-127L

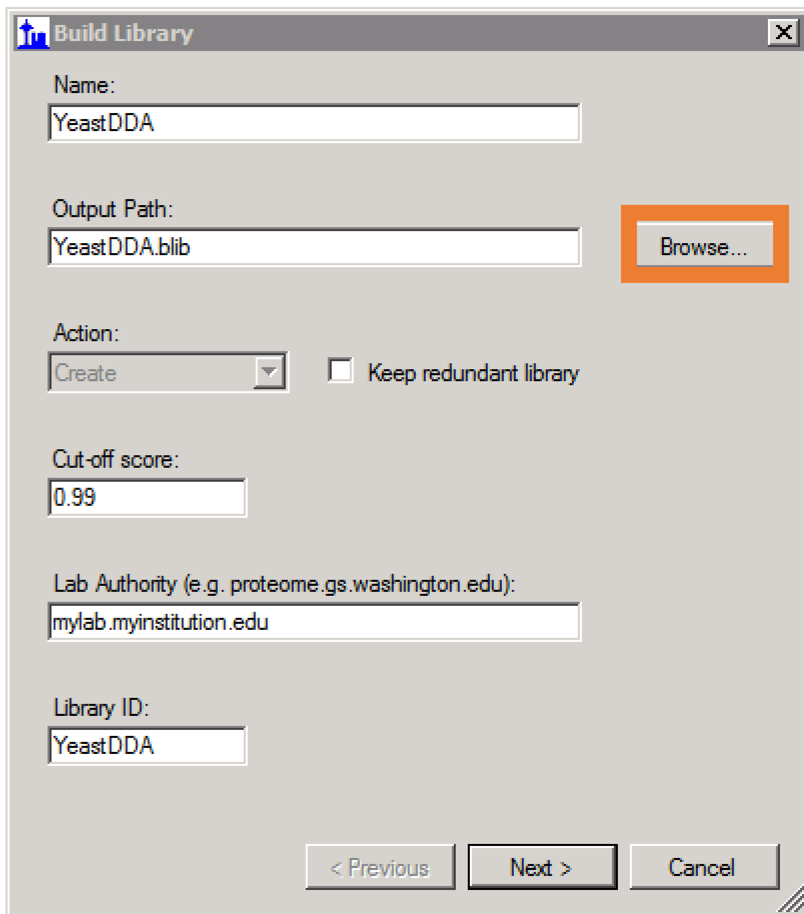
Edit List...

- [ ] Use DIA precursor window for exclusion

- [x] Auto-select all matching transitions

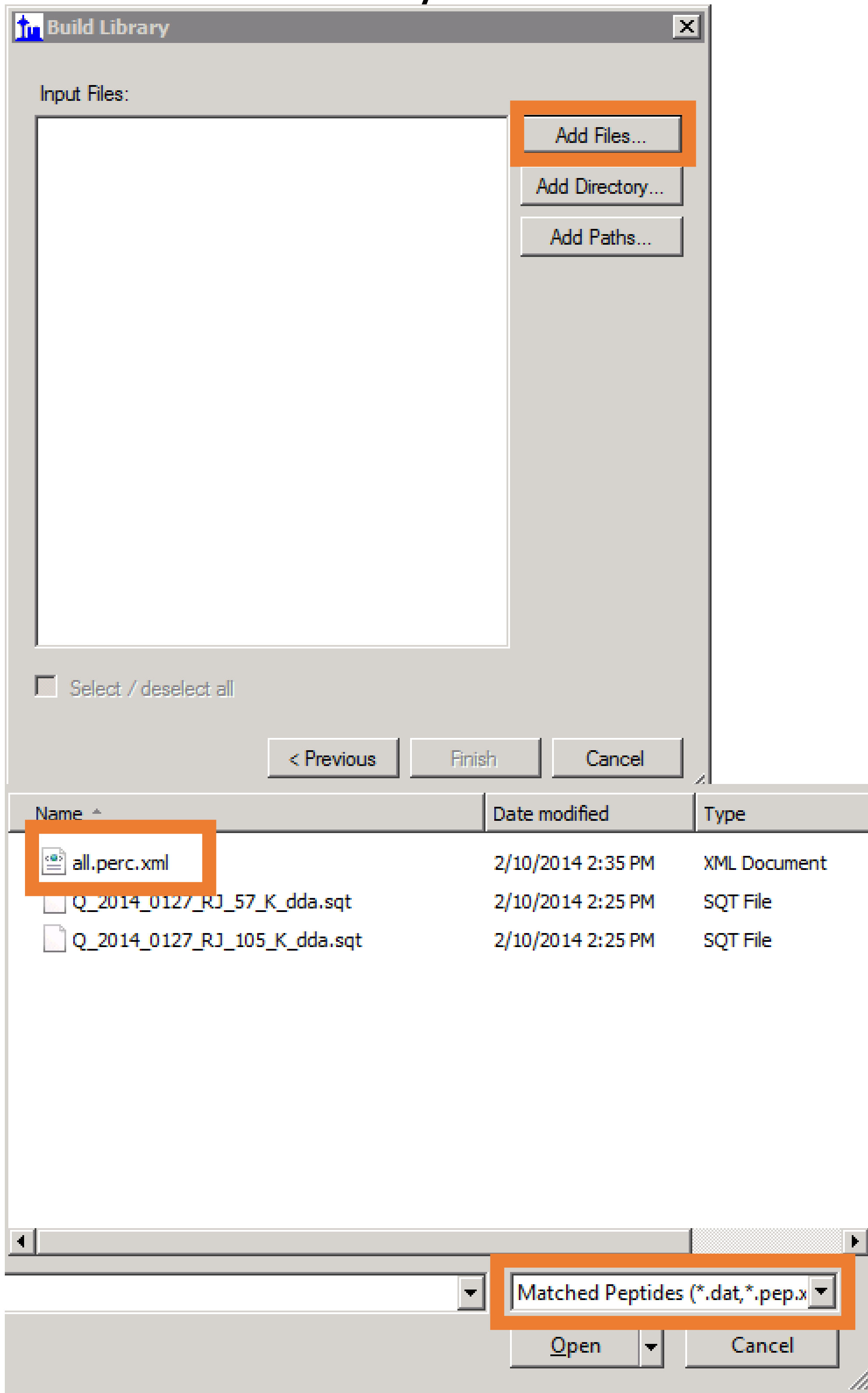
OK | Cancel

Settings -> Peptide Settings -> Library -> Build...



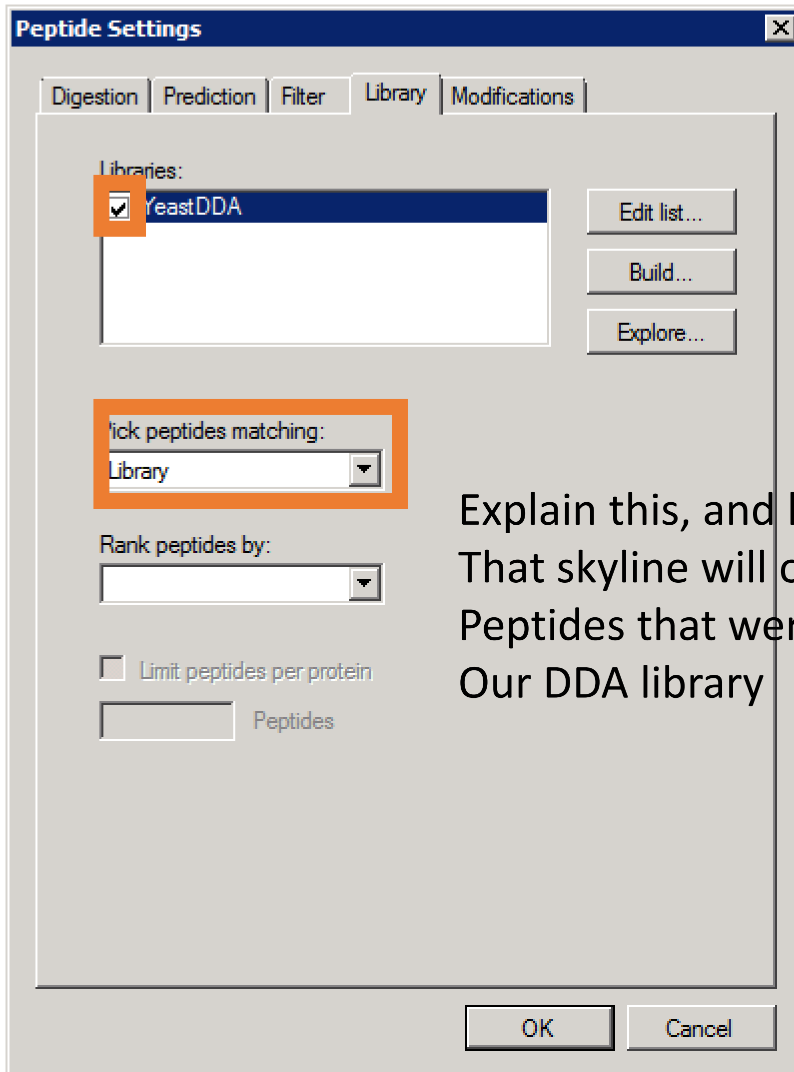
Point to spectralLibrary/YeastDDA.blib

Build library



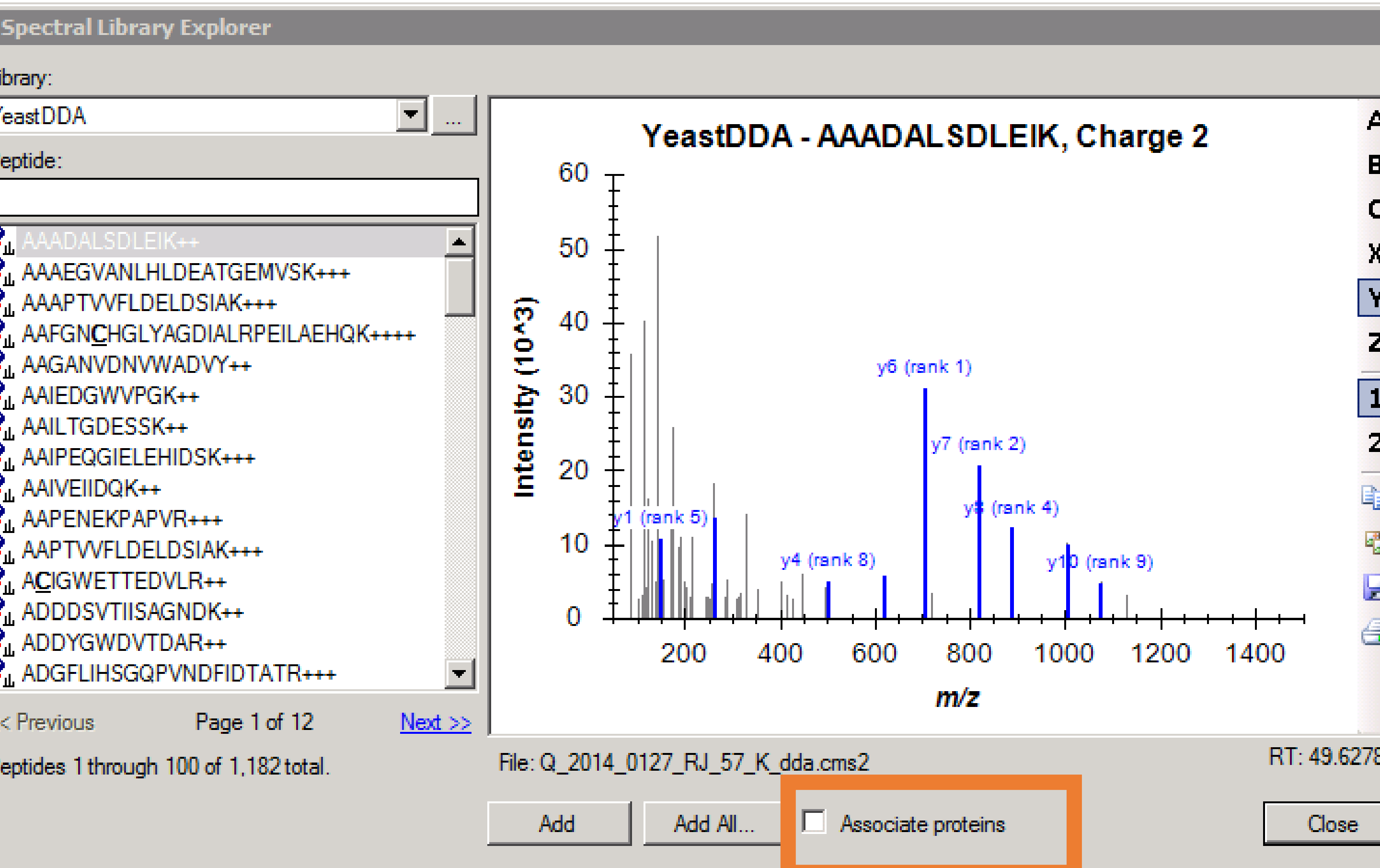
Click and hit all files to show all of the files in the directory

Settings → Peptide Settings → Library



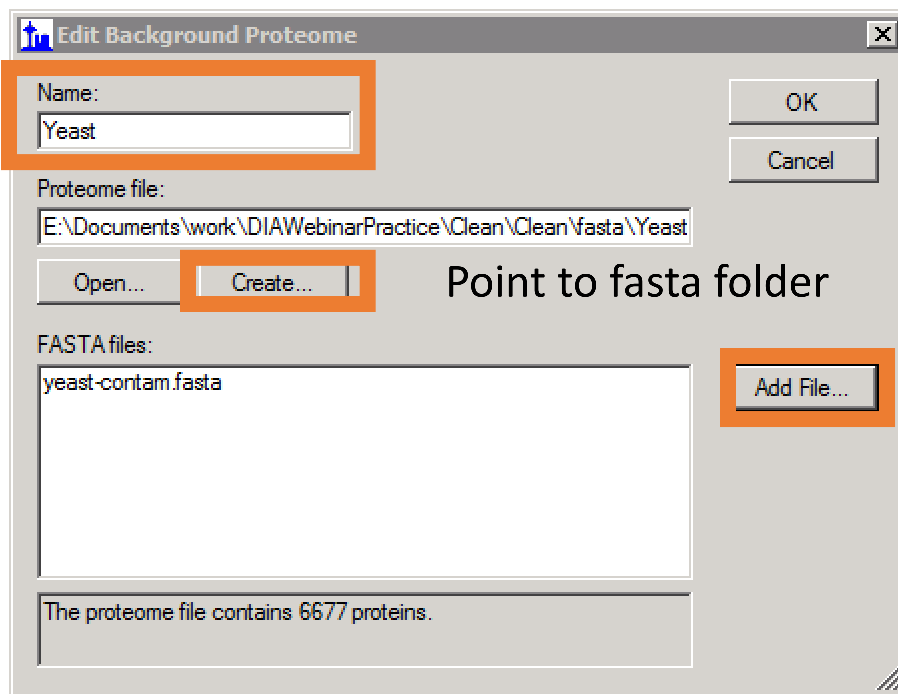
Explain this, and how it means
That skyline will only analyze
Peptides that were identified in
Our DDA library

View → Spectral Libraries



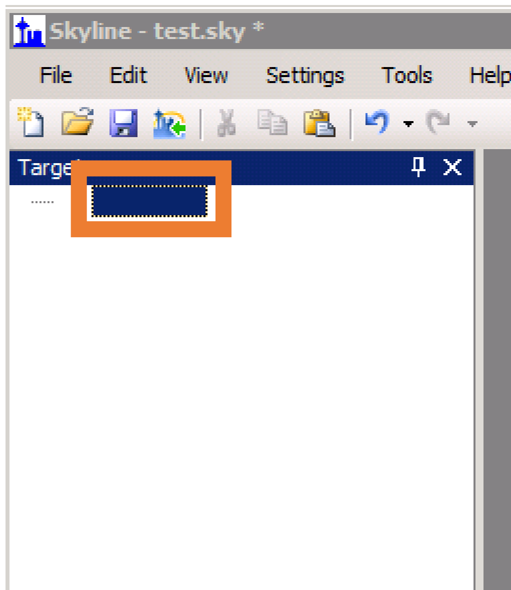
Mention we need to have the background proteome To associate peptides with proteins

Build a background proteome: Settings → Peptide Settings → Digestion (change max missed cleavages to 2)



Click OK → OK to exit out of peptide settings box

Inserting Proteins in the Main Window



Click here and start typing:

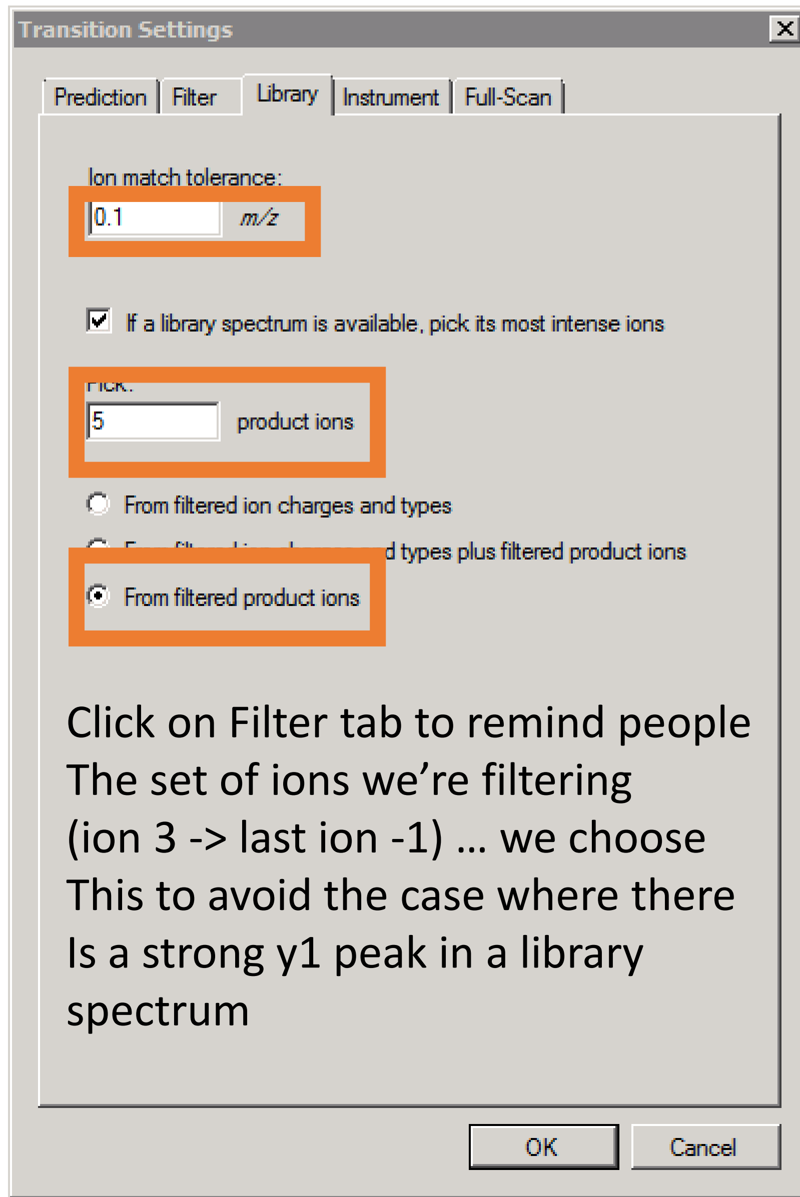
Insert "pyruvate decarboxylase" (YLR044C)

Type "glycolysis" and insert YCR012W

Explain the protein → Peptide → Transition Hierarchy

Show library spectrum

Settings → Transition Settings → Library



Show the transitions lists with 5 transitions now for a few peptides, then delete all

Inserting Proteins by name
Edit → Insert → Proteins

Pyruvate decarboxylase – works (use YLR044C)

Click on some of the peptides to show the spectra
Show precursors and transitions. (If they don't show up click View → Library Match)

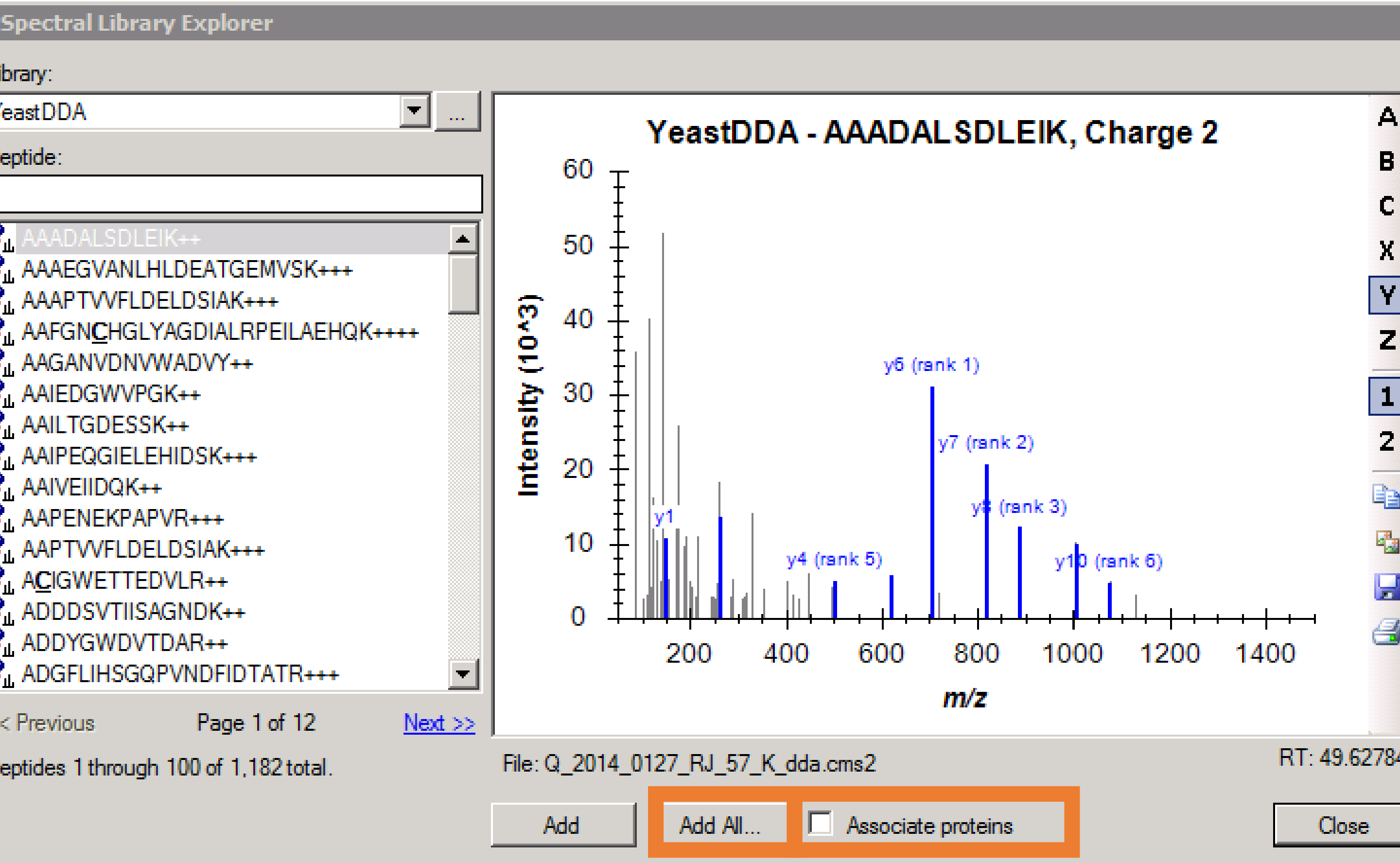
Try TOR1 and show how it doesn't insert

Because there aren't any peptides that were identified in
Our spectral library

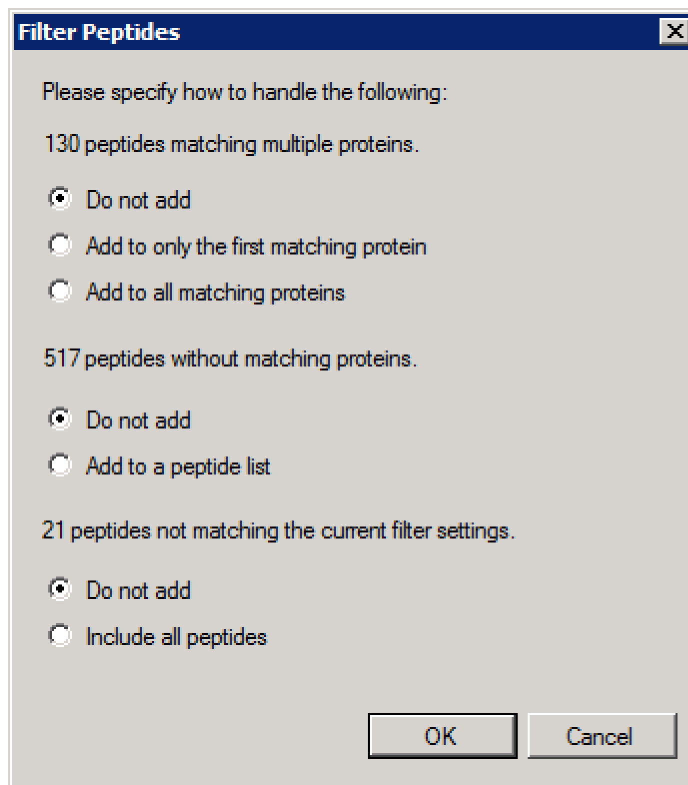
CONTEXT

- We are now going to insert all of the peptides in the DDA library

View → Spectral Libraries



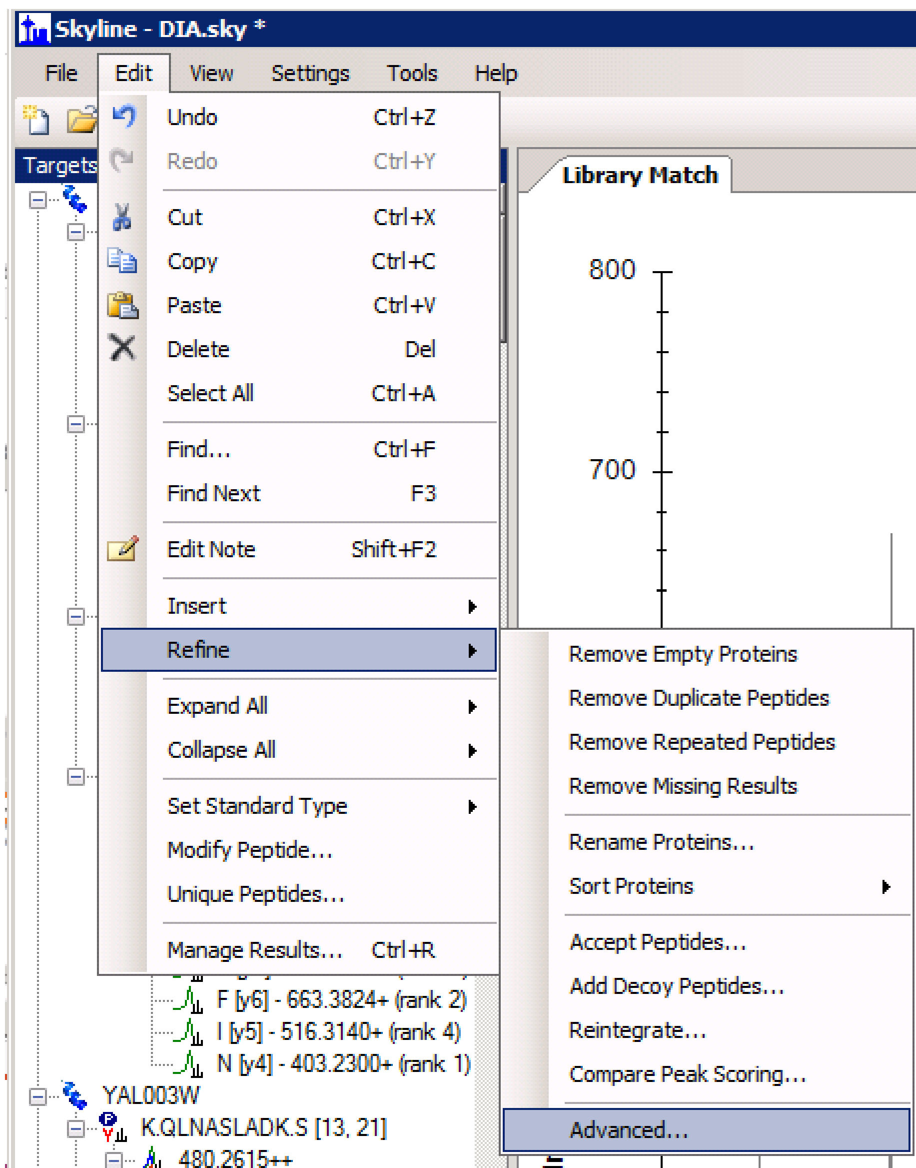
Explain these



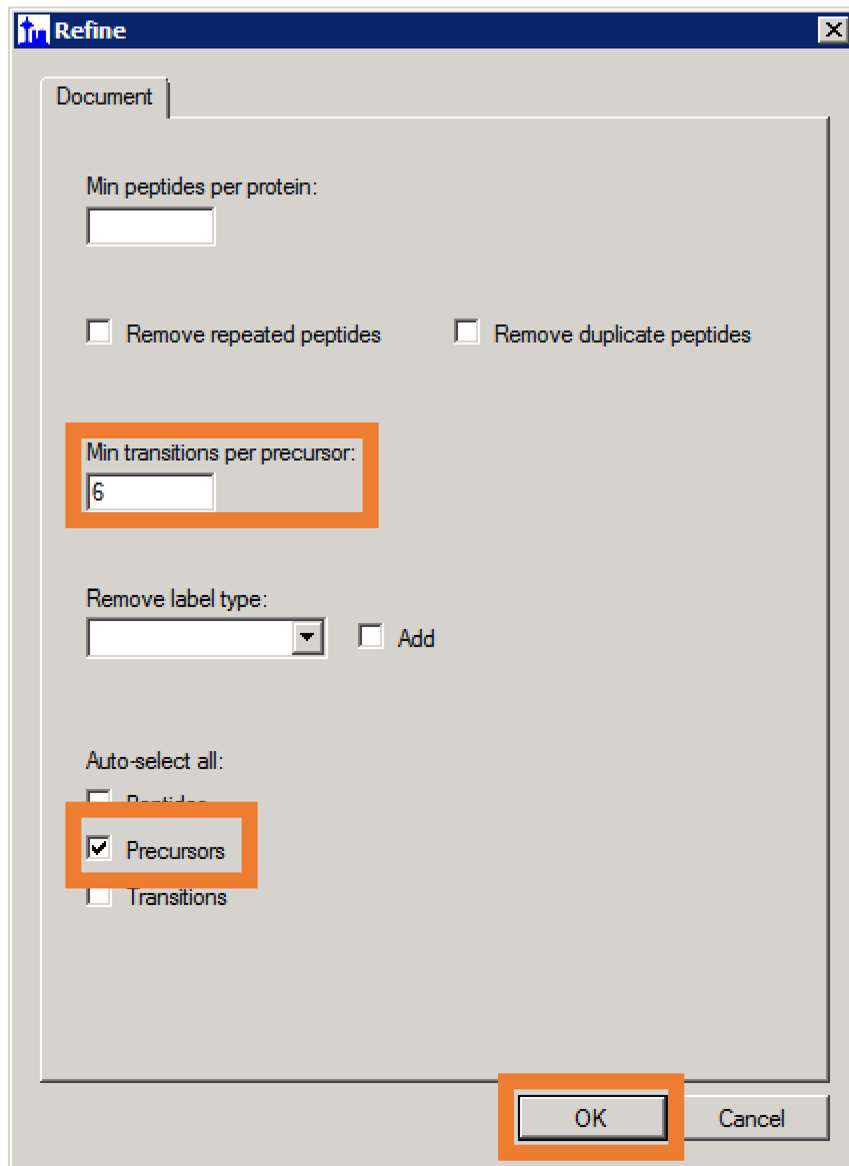
After this, delete trypsin contaminant

Show how some of the peptides have no product ion transitions, or a low number

Ctrl F for peptide SDVIFPILIPT

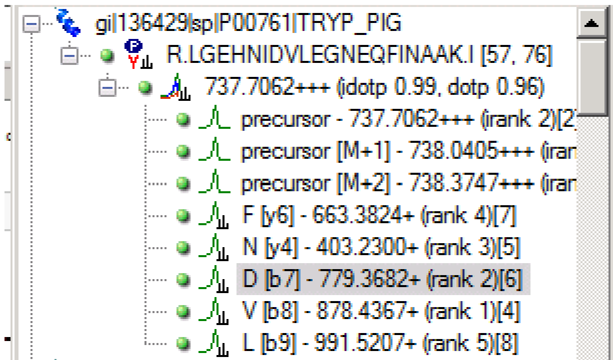


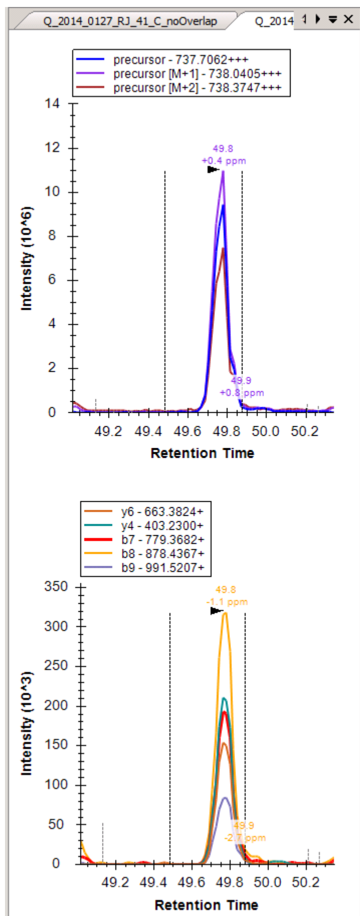
Refine to only keep peptides with 3 or more product transitions



Do ctrl-Z and ctrl-Y to show before and after precursor count

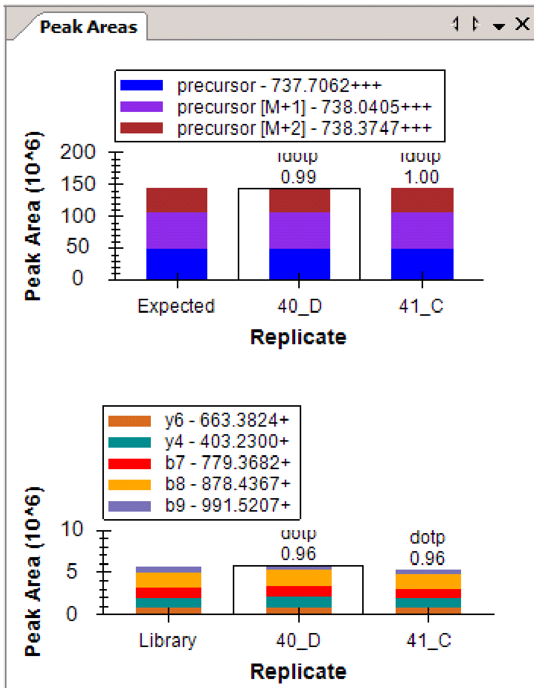
Import Results – 40_D_noOverlap 41_C_noOverlap



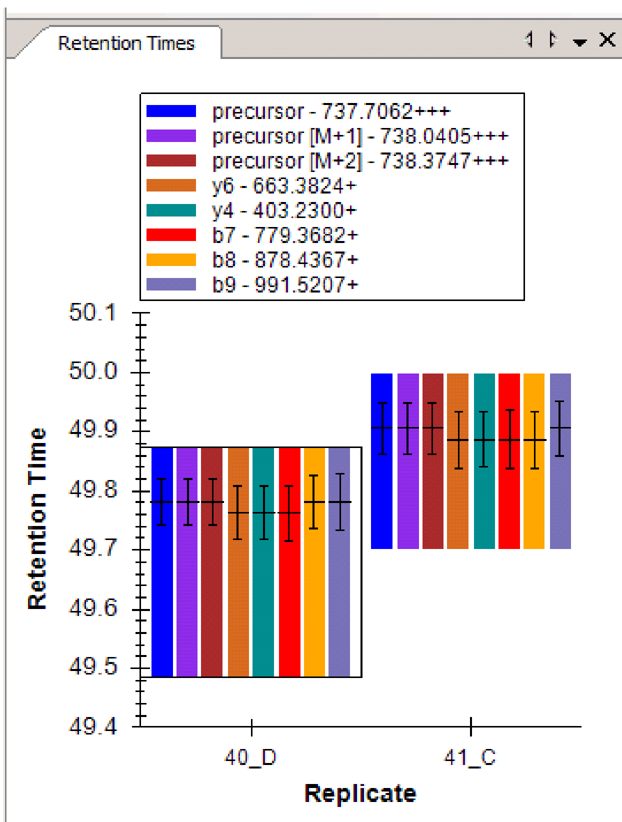


1. **Ctrl + F -> YAL038W**
2. Switch peptides, show graphs changing
3. Split View
4. Auto Zoom
5. Integration Boundaries
6. Show Peptide IDs from Other Run
7. Click on transitions, show highlighting on graph
8. Explain ranking on transitions
9. PPM Error
10. Show spectrum
11. Zoom out to see more of spectrum

Import Results – Peak Area and Retention Time



1. Show peak area replicate graph
2. Live graph – demonstrate by adjusting integration boundaries

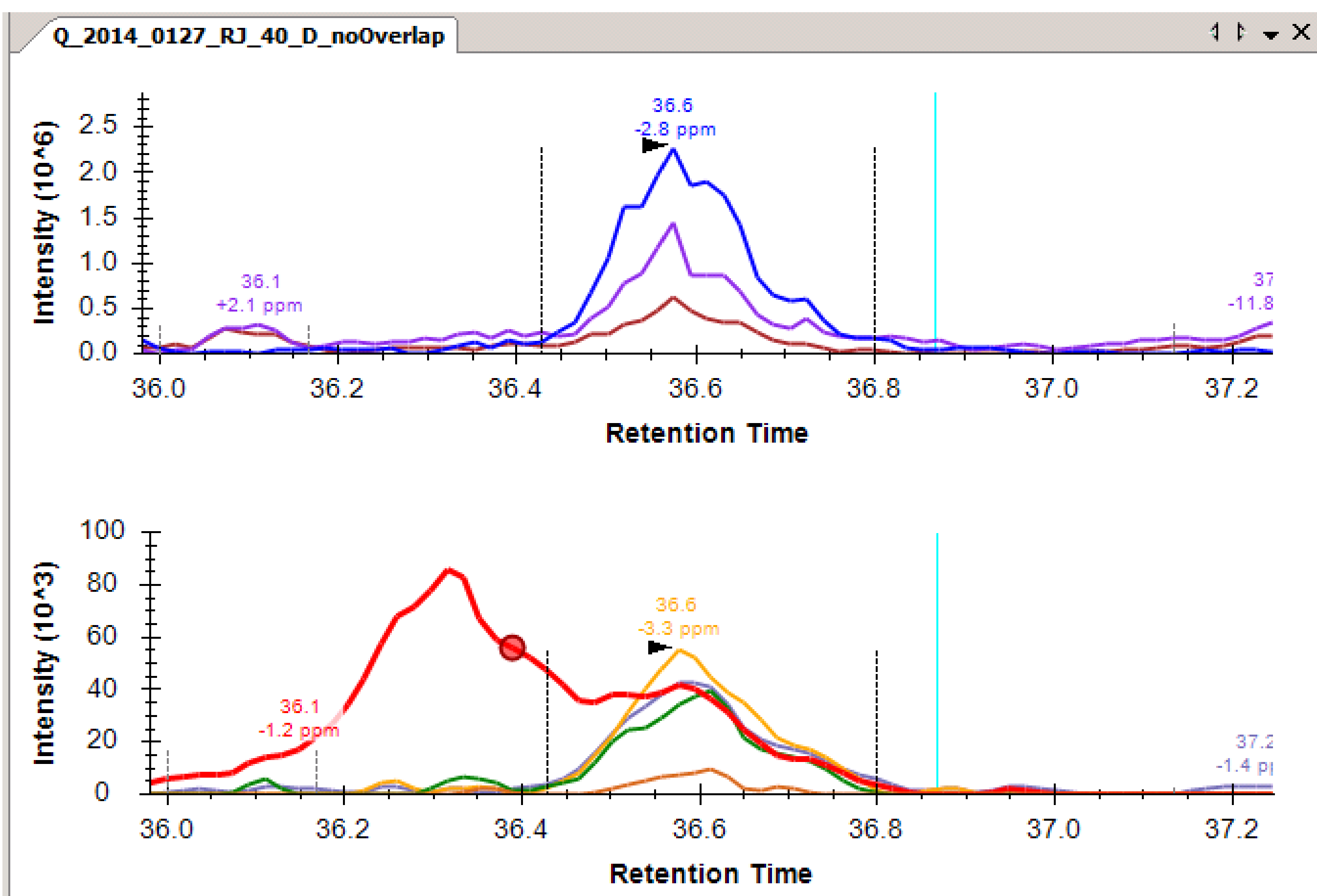


1. Show retention time replicate graph
2. Explain the meaning of the bars (center and FWHM of peak)
3. Click on other peptides to show how these graphs update

1. Move all graphs into document as panes and click View → Arrange Graphs → Tiled

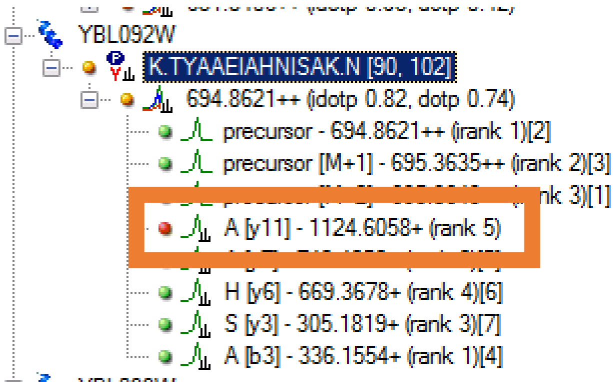
Manual refinement of transitions

1. Find a transition with interference (YAL038W – **SNLAGKPVIC**)
2. Show the spectrum from it
3. Delete the transition

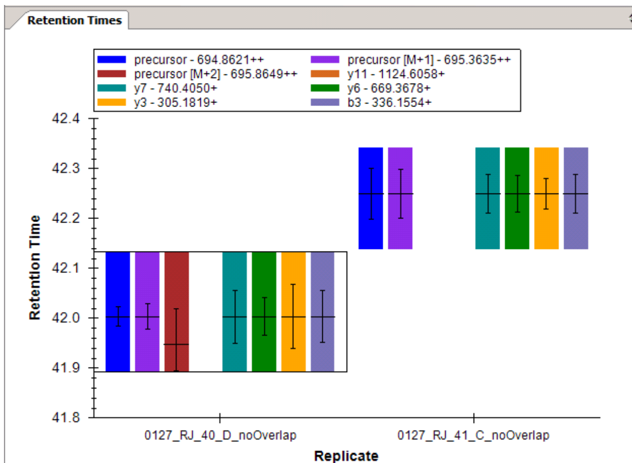


Integrate All Demonstration

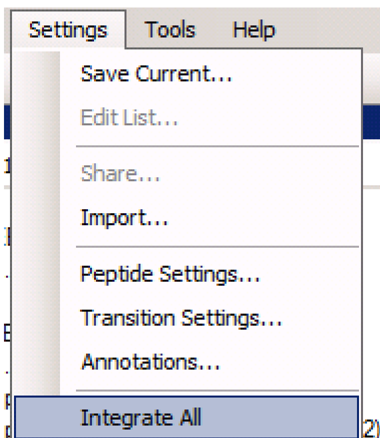
Ctrl -> F **-- TYAAEIAHNISAK**



y11 is a "misbehaving transition"



It doesn't show up in the retention time graph
And won't be integrated unless….



Settings –> Integrate All

Results Grid Demonstration

View → Results Grid

Click on a protein, then a peptide, then a precursor, then a transition

Click back on the precursor, change integration boundaries, and show resulting change in area on results grid.

Right click in the results grid and click choose columns

Add "Total Area MS1"

Add "Total Area Fragment"

Remove some of the columns on the right

Click around to show how it stays updated

Report Export

- Replicates
  - **Replicate Name**
- Proteins
  - **Protein Name**
- Proteins → Peptides
  - **Peptide Modified Sequence**
- Proteins -> Peptides -> Precursors
  - **Precursor Charge**
- Proteins -> Peptides -> Precursors -> Precursor Results
  - **Total Area MS1**
  - **Total Area Fragment**

Hit Preview
Enter View Name – Test Report
Export Test Report to reports directory
Open in Excel