# Supplementary Note 1: Analyzing pulseSILAC-DIA data with Prosit+EncyclopeDIA and Skyline

This tutorial is a practical guide for how to use the Encyclopedia software suite to build. . In this tutorial, we will detail how we analyzed the pulse SILAC-DIA data using EncyclopeDIA to first build a library of detected endogenous peptides, then using Skyline to pair the endogenous precursors to their "heavy" SILAC counterpart and extracting their chromatograms for quantification.

### SUMMARY: Four steps for pulseSILAC-DIA analysis

1. Convert .raw files to .mzML using MSConvert

\*Not covered here, please see Pino et al 2020 Supplementary Note 1 (<u>https://doi.org/10.1074/mcp.P119.001913</u>) for a detailed tutorial.

- 2. Build time point zero library using Prosit and EncyclopeDIA
- 3. Search pulsed data with library from step 2 using EncyclopeDIA
- 4. Extract light/heavy chromatograms using Skyline

### Citations

MSconvert (https://www.nature.com/articles/nbt.2377)

A cross-platform toolkit for mass spectrometry and proteomics. Chambers MC et al. *Nat Biotech* 30, 918-920 (2012). doi.org/10.1038/nbt.2377

Encyclopedia (<u>https://www.nature.com/articles/s41467-018-07454-w</u>)

Chromatogram libraries improve peptide detection and quantification by data independent acquisition mass spectrometry. Searle BC et al. *Nat Comm* 9, 5128 (2018). doi.org/10.1038/s41467-018-07454-w

Prosit+Encyclopedia (<u>https://www.nature.com/articles/s41467-020-15346-1</u>)

Generating high quality libraries for DIA MS with empirically corrected peptide predictions. Searle BC et al. *Nat Commun* 11, 1548 (2020). https://doi.org/10.1038/s41467-020-15346-1

You will need:

- MSConvert from Proteowizard: Windows only!
  - <u>http://proteowizard.sourceforge.net/download.html</u>
- EncyclopeDIA suite (\*.jar file): command line and cross-platform GUI
  - <u>https://bitbucket.org/searleb/encyclopedia/wiki/Home</u>
- Skyline: Windows only!
  - <u>https://skyline.ms/project/home/software/Skyline/begin.view</u>

To exactly replicate the results here, you will also need:

- RAW DIA data files from the tutorial bortezomib dataset (PXD022659)
- Ready-made predicted spectral library in \*.dlib format and accompanying FASTA of the Uniprot human reference proteome (reviewed; 20,350 entries) available on the Prosit Libraries website

# BUILD THE POOL OF PEPTIDES PRESENT AT TIME ZERO

1. Launch EncyclopeDIA. Under "Parameters:" click the "Library:" Edit button. Navigate to and select your Prosit predicted library (in DLIB format). Then, click the "Background:" Edit button to navigate to and select the corresponding FASTA for that Prosit library.

**! NOTE**: for a detailed tutorial on generating custom Prosit libraries, please see the Supplementary Info for Searle et al 2020

**! NOTE**: Prosit libraries for common organisms (human and yeast) can also be found on the <u>Prosit website here.</u>

EncyclopeDIA: Peptide Searching for DIA File View Convert Help					>
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2. At the top next to "Jobs:", click "Add MZML" to select the narrow window, gas phase fractionated (GPF) MZML files for your experiment.

**! NOTE**: The GPF library should be composed of the "time zero" sample from your pulse SILAC experiment. In other words, this pooled library sample should be composed entirely of endogenous/light peptides prior to incorporation of heavy SILAC amino acids.

EncyclopeDIA: Peptide Searching for DIA						- □ >
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3. After all GPF library files have completed, click "Save Chromatogram Library" to perform the final FDR filtering and save the ELIB.

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## SEARCH PULSED DATA WITH LIBRARY FROM TIME ZERO

4. Close and relaunch EncyclopeDIA to clear the cache. Under "Parameters:" click the "Library:" Edit button. Navigate to and select the chromatogram library you created in Step 3 above (in ELIB format). Then, click the "Background:" Edit button to navigate to and select the corresponding FASTA for the original Prosit library.

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Data Acquisition Type: Non-Overlapping DIA	~				
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5. At the top next to "Jobs:", click "Add MZML" to select the wide window, single-shot MZML files for your experiment.

#### FncyclopeDIA: Peptide Searching for DIA

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6. After all wide window, single-shot files have completed, click "Save Quant Report" to perform the global retention time alignment, fragment ion refinement, and FDR filtering; and save the ELIB.

EncyclopeDIA: Peptide Searchi	ing for DIA
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Linzyme		2382	Read 20200828_QEHFX_kp	pSILAC-DIA_btz_04_DIA_1	53.mzML	Wrot	e 16792 peptides identified at 1.	0% FDR
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## QUANTIFY SILAC PAIRS USING SKYLINE

7. Launch Skyline and open a blank document. Ensure that you are in protein mode and default settings (Settings > Default).



 Prepare the Skyline document with the appropriate settings per the EncyclopeDIA search performed above and as dictated by the instrument method settings. For this experiment, match the following parameters in Settings > Transition Settings:

Transition Settings	×	Transition Settings	×
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Use optimization values when pre	esent	TMT-128H     Edit List       TMT-129H     TMT-129H       TMT-130L     TMT-130H       TMT-131     V	
		☐ Use DIA precursor window for exclusion ☑ Auto-select all matching transitions	
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Transition Settings	×
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Min m / 7 Max m / 7	MS1 filtering
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Method match tolerance m/z: 0.005 m/z	Peaks: Resolution:
Firmware transition limit: Firmware inclusion limit:	Isotope labeling enrichment:
Marking Marking	MS/MS filtering
	DIA Centroided V
	Isolation scheme: Mass Accuracy: Results only V 10 ppm
	Use high-selectivity extraction
	Retention time filtering
	Use only scans within 5 minutes of predicted RT
	O Include all matching scans
OK Cancel	OK Cancel

- Prediction: Precursor/Product ion mass="Monoisotopic"
- Filter: Ion charges=1,2 Ion types="y"\*, From=Ion 3, To=last ion, no special ions

- Library: Ion match tolerance=0.005 m/z, check "If a library spectrum is available, pick its most intense ions", pick=5 product ions, select "From filtered product ions"
- Instrument: "Min m/z=50, Max m/z=1500, Method match tolerance m/z=0.005
- Full-Scan (MS1): "Isotope peaks included=None"
- Full-Scan (MS/MS): "Acquisition method=DIA, Product mass analyzer=Centroided, Isolation scheme=Results only, Mass Accuracy=10 ppm, check "Use only scans within "2" minutes of MS/MS IDs
- Next, set the parameters for the Prediction and Filter tabs of the peptide settings (Settings > Peptide Settings), again per the EncyclopeDIA search performed above and as dictated by the instrument method settings. For this experiment:

- Prediction: Check "Use measured retention times when present", Time window=2
- Filter: Min length=3, Max length=40, no excluded amino acids checked
- 10. In the Digestion tab of the Peptide Settings (Settings > Peptide Settings > Digestion) expand the "Background proteome:" drop down to select <Add...>. Type a working name for this background proteome and then click "Create..." to give the background proteome file a name and filepath location. Click "Add..." and navigate to the same FASTA used in steps 1 and 4 above. Once the background proteome file has been created, press "OK" to return to the Peptide Settings window.

**! NOTE**: After selecting the FASTA, this may take some time. Skyline should display a progress bar.

Peptide Set	tings					×			
Peptide Set Digestion Enzy Tryp Max 1 0 Back Non Enfor	tings Prediction me: sin [KR   P] missed cleave composition roce peptide un e	Filter ages: ome:	Library ~ ss by:	Modifications	Quantification	×	Edit Background Proteome         Name:         tumover_bortezomib         Proteome file:         Open         Create         FASTA files:	OK Cancel Add File	×
				OK	Cancel		Click the 'Open' button to choose an existing proteome file, or click the 'Create' button to create a new proteome file.		

11. In the Library tab of the Peptide Settings window (Settings > Peptide Settings > Library) click "Edit list..." and then "Add..."

igestion Prediction Filter Library Mod	lifications Quantification	💁 Edit Libraries	×
Libraries:	Edit list	Libraries:	
	Build		Add
	Explore		Copy
			Edit
			Remove
			Up
			Down
			Reset

12. Fill out a working name for the library, then next to "Path:" click "Browse..." and navigate to the final ELIB that was saved in step 6 above. Select "OK" and then "OK" again to get back to the Peptide Settings window. Check the box next to the library that was just set up, ensure that the dropdown for "Pick peptides matching:" has "Library" selected, and then click "Explore..."

**! NOTE**: After clicking "Explore..." a pop-up may appear notifying that "Peptide settings have been changed. Save changes?" Click YES

	Quantification	Digestion Prediction Filter Ubrary Modifications Quantific	ation
Libraries:		Libraries:	
🌆 Edit Libraries	×	tumover_bortezomib Edt list	t
15mm		Build.	
dit Library	×	Explore	e
Name:	OK		
tumover_bortezomib			
	Cancel		
Path:		Pick peptides matching:	
C:\Users\linds\Google Drive\Penn\proj\silac_dia\	Browse	Library V	
Spectral Library Links:		Rank peptides by:	
Spectral Library Links: PeptideAtlas <u>NIST GPM</u>		Rank peptides by:	
Spectral Library Links: <u>PeptideAtlas NIST GPM</u> Use explicit peak bounds		Rank peptides by:	
Spectral Ubrary Unks: <u>PeptideAtlas NIST GPM</u> Use explicit peak bounds		Rank peptides by:	
Spectral Library Links: PeptideAtlas NIST GPM Use explicit peak bounds OK Can	cel	Rank peptides by:	
Spectral Library Links: <u>PeptideAtlas NIST GPM</u> Use explicit peak bounds OK Can	cel	Rank peptides by:	
Spectral Library Links: <u>PeptideAtlas</u> <u>NIST GPM</u> Use explicit peak bounds OK Can	cel	Rank peptides by:	

13. Once the spectral library explorer window is launched, check the box at the bottom to "Associate proteins" and then click "Add all..."

**! NOTE**: After clicking "Add all...", a popup progress bar for "Matching peptides to the current document settings" should appear. This may take some time.



14. When the protein association is complete, a popup window should appear with options for how to handle certain peptide-to-protein mapping situations. Skyline does not use protein groups, so "Add to all matching proteins" is recommended for downstream analysis to ensure that peptide mapping is possible. Click "OK". After a moment, another popup will appear describing the final results. Select "Add All". Close out of the spectral library explorer.

Skyline-daily		×
This operation will add 4085 and 65187 transitions to the	proteins, 20258 peptides, 20258 document.	8 precursors
Pa	Add All	Cancel

15. The left-hand "Target List" pane should now be populated with proteins, but all these proteins are light/endogenous. Go to Settings > Peptide Settings > Modifications. Under "Isotope modifications", check the box corresponding to the experiment SILAC labeling scheme, here "heavy" 13C(6)15N(4) arginine and "heavy" 13C(6)15N(20) lysine. Under the "Isotope label type" dropdown, select "heavy". Click OK to exit out of the Peptide Settings window.

**! NOTE**: Less conventional or custom isotope modifications can be added with Edit list > Add > Edit Isotope Modifications.

gesuon	Prediction	Filter	Library	Modifications	Quantification
Struc	tural modifica	ations:			
	Carbamidome Phospho+Pro Methyl + Prop Acetyl + Prop	thyl (C) pionyl ionyl ionyl	^	Edit list.	
	Dimethyl + Pr	opionyl	~		
Max	variable mod	s:	Max losse	es:	
3		1	1		
hear	pe label type vy	;	~	•	
Isoto	pe modificatio	ons:			
	Label:13C(6) Label:13C(5) Label:13C(5) Label:13C(5) Label:13C(6) Label:13C(6)	15N(1) (l 15N(1) (l 15N(1) (f 15N(1) (f 15N(4) (c 15N(2) (c	-) /) P) Cterm F Cterm K ~	Edit list.	
Inter	nal standard t	ype:			
			~		

16. Navigate to Refine > Advanced and under the "Remove label type" options, check the box next to "Add" and select "heavy" from the dropdown. Click "OK".

Refine						×
Document	Group Compariso	'n				
Min pep	tides per protein:					
Ren	iove repeated pep iove peptides miss	tides ing library	match	Remove	duplicate pe	ptides
Min tran	sitions per precurs	or;				
Add labe	el type: ~	🗹 Ad	ld			
Auto-sel	ect all: tides					
Prec	sursors isitions					
					ок	Cancel

17. Save the Skyline document. Then, choose File > Import > Results to add single-injection replicates and click OK. Navigate to and select all the single-shot, wide-window MZML files used in the EncyclopeDIA analysis above. Choose whether to shorten the file names or not, click "OK", and the import should begin.

**! NOTE**: The chromatogram import graphic should appear and can be used to track progress. This may take time.

mport Results		~					
Add single-injection replicates in files	5	ОК					
Optimizing:		Cancel					
None ~		Cance					
<ul> <li>Add multi-injection replicates in direct</li> </ul>	tories						
Name:							
<ul> <li>Add files to an existing policate</li> </ul>							
Name:							
	~						
	άβ.						
Files to import simultaneously:							
Files to import simultaneously:         Many         Show chromatograms during import         Retry after import failure							
Files to import simultaneously:         Many         Show chromatograms during import         Retry after import failure         mporting Results	202008	28 OFHEX II		DIA htz	02 DIA 1	29	
Files to import simultaneously:         Many         Show chromatograms during import         Retry after import failure         mporting Results         1. 20200828_QEHFX_lk         13%	202008 16 †	28_QEHFX_II	kp_pSILAC	-DIA_btz_	02_DIA_1:	29	
Files to import simultaneously:         Many         Show chromatograms during import         Retry after import failure         mporting Results         1. 20200828_QEHFX_lk         Cancel         14%	202008 16 +   14 +	28_QEHFX_II	kp_pSILAC	-DIA_btz_	02_DIA_1:	29	
Files to import simultaneously: Many  Show chromatograms during import Retry after import failure  1. 20200828_QEHFX_lk 1. 20200828_QEHFX_lk Cancel 1. 44% Cancel 1	202008	28_QEHFX_II	kp_pSILAC	-DIA_btz_	02_DIA_1:	29	
Files to import simultaneously: Many Show chromatograms during import Retry after import failure mporting Results 1. 20200828_QEHFX_k Cancel 13% Cancel 14% Cancel 14% Cancel 14% Cancel 14% Cancel	202008	28_QEHFX_II	¢p_pSILAC	-DIA_btz_	02_DIA_1:	29	
Files to import simultaneously: Many  Show chromatograms during import Retry after import failure  mporting Results  1. 20200828_QEHFX_lk Cancel 14% Cancel	202008 16 + 14 + 12 + 12 + 10 + 12 + 10 + 12 + 10 + 12 + 10 + 10 + 12 + 10 +	28_QEHFX_II	kp_pSILAC	-DIA_btz_	02_DIA_1:	29	
Files to import simultaneously: Many Show chromatograms during import Retry after import failure mporting Results 1. 20200828_QEHFX_Ik Cancel 13% Cancel 14% Cancel 14% Cancel 14% Cancel 14% Cancel 5. 20200828_QEHFX_Ik	202008 16 + 14 + 12 + 10 +	28_QEHFX_II	¢p_pSILAC	-DIA_btz_	02_DIA_1:	29	
Files to import simultaneously: Many Show chromatograms during import Retry after import failure mporting Results 1. 20200828_QEHFX_k 2. 20200828_QEHFX_k Cancel 14% 3. 20200828_QEHFX_k Cancel 14% Cancel 14% Cancel 6. 20200828_QEHFX_k 6. 20200828_QEHFX_k	202008 16 + 14 + 12 + 10 +	28_QEHFX_II	¢p_pSILAC	-DIA_btz_	02_DIA_1:	29	
Files to import simultaneously: Many Show chromatograms during import Retry after import failure mporting Results 1. 20200828_QEHFX_Ik 2. 20200828_QEHFX_Ik Cancel 14% 3. 20200828_QEHFX_Ik Cancel 14% 14% 14% 14% 14% 14% 14% 14%	202008 16 + 14 + 12 + 10 +	28_QEHFX_II	¢p_pSILAC	-DIA_btz_	02_DIA_1:	29	
Files to import simultaneously: Many Show chromatograms during import Retry after import failure mporting Results 1. 20200828_QEHFX_Ik 2. 20200828_QEHFX_Ik Cancel 14% Cancel 14% Cancel 14% Cancel 6. 20200828_QEHFX_Ik 6. 20200828_QEHFX_Ik 7. 20200828_QEHFX_Ik 8. 20200828_QEHFX_Ik	202008 16 14 12 10 10 10 10 4 2 0 4 4 2 0	28_QEHFX_I	(p_pSILAC)	-DIA_btz_	02_DIA_1:	29	
Files to import simultaneously:   Many   Show chromatograms during import   Retry after import failure   mporting Results   1. 20200828_QEHFX_Ik   1. 20200828_QEHFX_Ik   2. 20200828_QEHFX_Ik   2. 20200828_QEHFX_Ik   2. 20200828_QEHFX_Ik   Cancel   14½   Substant   Cancel   14½   Cancel   14½   Cancel   14½   Cancel   14½	202008 16 14 12 10 10 10 4 2 0 0 0 2	28_QEHFX_II	<p_psilac< td=""><td>-DIA_btz_ 80</td><td>02_DIA_1:</td><td><b>29</b> + 1 + 120</td><td></td></p_psilac<>	-DIA_btz_ 80	02_DIA_1:	<b>29</b> + 1 + 120	

18. Paired SILAC peptides can now be viewed in Skyline. Quantifications can be exported using File > Export > Report...