Limited Proteolysis Coupled to Mass Spectrometry (LiP-MS)

1. Rationale/Aim

Limited proteolysis coupled to mass spectrometry (LiP-MS) is a structural proteomics method that profiles structural changes in complex proteomes in response to small molecules or other perturbations. The assay relies on the limited proteolysis using the sequence-unspecific protease proteinase K (PK). Small molecule binding can lead to protein structural changes such as occupying the binding site, changes in protein rigidity, and changes in tertiary and quaternary structure. These structural changes lead to treatment-dependent differences in flexibility and accessibility of the protein, which in turn is captured by the change in cleavage sites of PK. Thus, the change in abundance of peptides generated by PK in the different conditions can be used to identify regions where structural changes occur, providing a quantitative mass spectrometry-based readout for protein structural changes.

The following protocol is used for target engagement analysis in complex lysates. The protocol can easily be adapted for LiP-MS of low complexity target protein samples. First, proteins must be natively extracted from the cells to ensure intact protein structures. Then, the drug is added for a short amount of time (typically five minutes) which is followed by the precisely timed limited-proteolysis step. The exact timing is important to ensure reproducibility in the PK cleavage patterns. Fragments generated by PK cleavage depend on the protein structure, which can be altered by the treatment. These fragments are processed further by reduction and protection of cysteines as well as a tryptic digest for subsequent mass spectrometry analysis. Note that the peptides generated by the tryptic digest still reflect the fragments generated by PK during limited proteolysis.

Besides providing an orthogonal readout for target deconvolution, LiP-MS can identify peptides that undergo structural changes upon probe binding and therefore can be used for structural proteomic fingerprinting of molecular probes.

2. Experimental conditions

2.1 Key Requirement:

To perform a simple drug spike-in LiP-MS experiment, the materials necessary are a native cell lysate (100 µg protein per sample, typically 2 conditions x 4 replicates are done → 800 µg protein in total) and the small molecule to be investigated (51 µM or 510 µM stock solution to add 1 µl per sample for a final concentration of 1 µM or 10 µM, typically for 1 treatment condition x 4 replicates → 4 µl total volume). The same volume of solvent is necessary for the control condition.

The key pieces of equipment include a PCR machine with at least two separate temperature controlled compartments, multichannel pipettes (for volumes of 1 µl and 5 µl), and a UPLC-MS setup capable of acquiring MS/MS data. We typically use Thermo Fisher Orbitrap instruments and measure in data-independent acquisition mode, although data-dependent acquisition also yields adequate data.

2.2 Key Resources Table:

Reagents (items)	*Suppliers*	*Cat. No.*
Chemicals		
HEK Flp-In™ T-REx™ 293 Cell Line	Invitrogen	#R78007
HEPES	Sigma	H4034
Magnesium chloride hexahydrate	Fluka	63072
Potassium chloride	Emsure	1.04936
Sodium hydroxide	Emsure	1.06498
Ammonium bicarbonate	Fluka	09850
BCA Protein assay kit	Pierce Thermo Scientific	23225
DMSO	Sigma	D2650
Rapamycin	LC laboratories	R-5000
DOC	Sigma-Aldrich	D6750
TCEP HCl	Thermo Scientific	20490
Iodoacetamide	Sigma	I1149-25G
Formic acid	Sigma	27001
Water, HPLC for gradient analysis	Fischer Brand	W/0106/17-4
Methanol, HPLC for gradient analysis	Fischer Brand	M/4058/17-4
Acetonitrile, HPLC for gradient analysis	Sigma-Aldrich	34851
Recombinant proteins		
Proteinase K from *Tritirachium album* (PK)	Sigma-Aldrich	P2308
Trypsin	Promega	V5113
Lysyl EndopeptidaseR (Lys-C)	FUJIFILM Wako Pure Chemical Corporation	125-05061
Kits		
Pierce™ BCA Protein Assay Kit	Thermo Fischer Scientific	23227
10x iRT Peptide Kit	Biognosys AG	Ki-3002-2
Other		
Pellet pestle with cordless motor	Sigma	Z359971
0.20 µm PVDF filter plates	Corning	3505
MicroSpin C18 plates	The Nest Group	SNS SS18V
C18 columns	The Nest Group	HMM S18V
MS sample vials	BGB	TPXCV1103K
Orbitrap Fusion Lumos Tribrid mass spectrometer	Thermo Fisher Scientific	
Aquity M-Class UPLC System Waters	Aquity M-Class UPLC System Waters	
Software		
SpectroMine version 2	Biognosys AG	
Spectronaut version 15	Biognosys AG	
Rstudio	Rstudio	
R 4.0.2	The R Foundation	

3. Protocol

3.1 Workflow

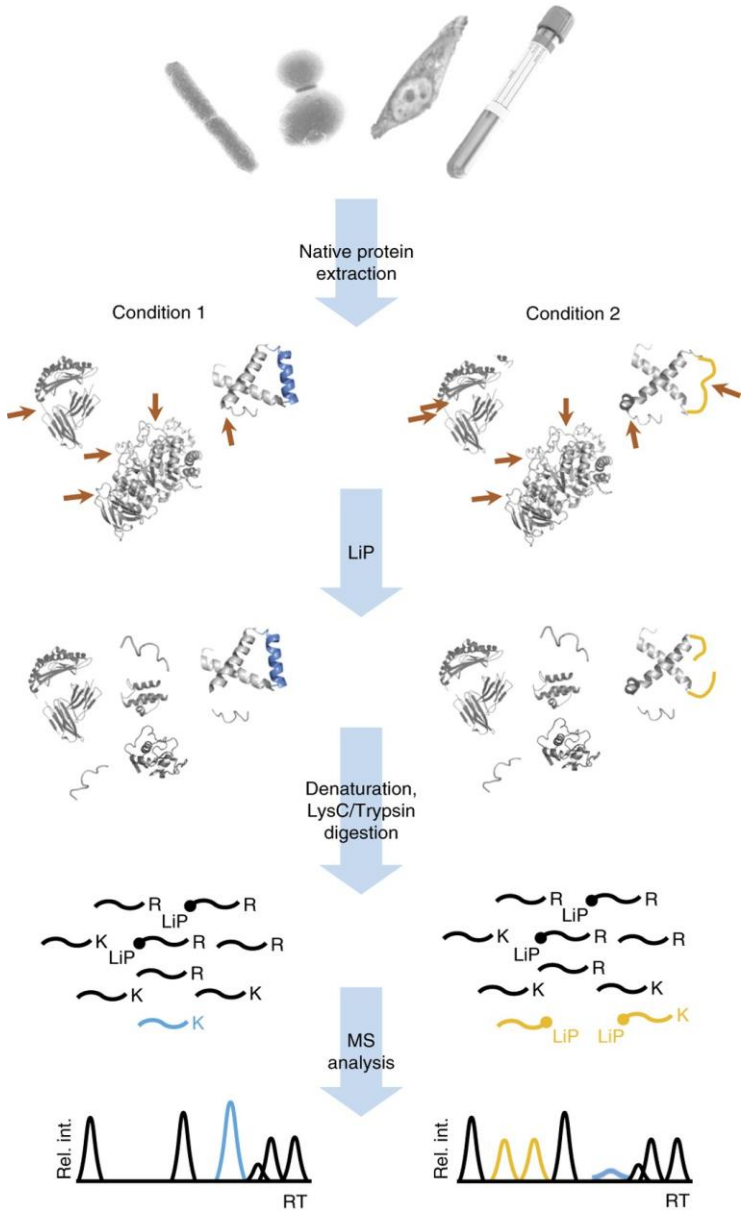
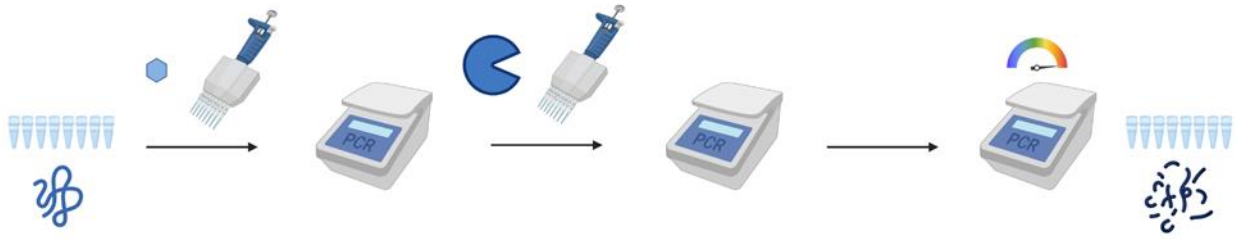


Figure from Schopper, S. et al. Nature Protocols 12, 2391–2410 (2017)



Detailed overview of LiP step. First, the drug is added to the lysate. The tubes are incubated at 25°C for five minutes. Subsequently, PK is added to the samples. The tubes are again incubated at 25°C for five minutes. The PK is then heat inactivated at 99°C for five minutes. The samples are cooled on ice afterwards (not shown).

3.2 Protocol:

1. Native lysis and dilution

1. Thaw the cell pellet on ice
2. Resuspend the cell pellet in 200 µl LiP buffer (1 mM MgCl2, 150 mM KCl, 100 mM HEPES) and transfer it to a 15 ml Falcon tube
3. Use an pellet pestle with a cordless motor to lyse cells in the Falcon tube using 10 pulses, 1 minute on ice, repeat 10 times
    a. Make sure to avoid warming and bubbles
    b. Clean the pestle before and after use in 70% EtOH and MilliQ water
4. To remove membranes and other debris, transfer the lysate to an Eppendorf tube and centrifuge at max speed for 15 minutes at 4°C
    a. Collect the supernatant
5. Determine the total protein concentration using a Pierce BCA protein assay kit according to manufacturer's instructions
    a. Keep lysate on ice
6. Dilute the lysate to 2 µg/µl and distribute 50 µl into each PCR tube on a strip for easy handling
    a. The total protein amount is 100 µg per replicate

2. LiP Step

1. Prepare a PCR tube strip containing the drug at 510 µM (e.g. rapamycin 10 µM final) or the solvent (example: DMSO) in alternating tubes
    a. Provide more than is necessary for easy pipetting
2. Prepare a PCR tube strip containing PK at a concentration of 0.2 µg/µl to ensure an enzyme:substrate ratio of 1:100
    a. Keep PK on ice!
    b. Provide more than is necessary for easy pipetting
3. Preheat the PCR machine to 25°C in one chamber and 99°C in another
4. Add 1 µl of the drug to the lysate and mix

   a. Mixing can be done by pipetting or by vortexing and quickly spinning down
5. Incubate at 25°C for five minutes
   a. Start the timer the second the drug is added to the lysate!
6. Add 5 µl of PK to the lysate and mix
   a. Mixing can be done by pipetting or by vortexing and quickly spinning down
7. Incubate at 25°C for five minutes
   a. Start the timer the second PK is added to the lysate!
8. Transfer the tubes to 99°C to inactivate PK
9. Transfer the tubes to ice to cool for at least five minutes

3. Tryptic digest

1. Prepare the following solutions:
   a. 10 % DOC (sodium deoxycholate) (w:v 200 mg/2 ml in water)
   b. 200 mM TCEP (5.8 mg / 100 µl HEPES 1 M)
   c. 1M IAA (37 mg/200 µl H_2O)
   d. 100 mM ABC (ammonium bicarbonate) (158 mg/20 ml H_2O)
2. Transfer the lysate to new Eppendorf tubes containing the same amount of 10% DOC (56 µl + 56 µl DOC)
3. Reduce disulfide bonds with a final concentration of 5 mM TCEP (112 µl + 2.87 µl TCEP)
4. Incubate 40 minutes at 37°C, 800 rpm on a thermomixer
5. Alkylate samples with a final concentration of 40 mM IAA (114.87 µl + 4.79 µl IAA)
6. Incubate 30 minutes at 30°C in the dark, at 800 rpm
7. Dilute sample with 100 mM ABC to a DOC concentration of 1% (1:5) (478.63 µl)
8. Add 1 µg Lys-C and 1 µg trypsin to each sample
9. Incubate overnight at 37°C and 800 rpm
10. Stop the digestion by adding 50% formic acid to reach a final concentration of 2% 1:25 (approx. 25 µl 50% FA)
11. Shake for 5 min at 37°C at 800 rpm
12. Remove precipitated DOC by filtration in fume hood using a 96 well vacuum manifold and 0.2 µm PVDF filter plates

4. C18 clean-up

1. Prepare the following solutions. Note to prepare buffers A and B using MS-grade H_2O and ACN
   a. Methanol
   b. Buffer A: 0.1% FA
   c. Buffer B: 50% ACN, 0.1% FA
2. Use C18 columns or C18 96-well plates
3. Wash C18 columns with 1 volume MeOH and 1 volume Buffer B
   a. Spin at 500 x g after each washing step
   b. Appropriately discard the waste
4. Equilibrate with 2 volumes Buffer A
   a. Spin at 500 x g after each step

5. Transfer Sample onto column collect flow-through
    a. Spin at 500 x g after each step
6. Wash with 3 volumes Buffer A
    a. Spin at 500 x g after each step
7. Elute 2 times with 100 µl Buffer B for a final volume 2400 µl
8. Dry samples in a speed vac at 45°C (2-3 h)
9. Store samples at -20°C until resuspension

5. Peptide resuspension

1. Resuspend peptides in Buffer A to a final concentration of 1 µg/µl
    a. We assume a 50% loss when starting out with 100 µg protein, so we resuspend in 50 µl
    b. Make sure to rinse walls of the tube with Buffer A
2. Shake the samples at 25°C for 5 minutes at 800 rpm
3. Sonicate for 5 min in a water bath sonicator at 25°C
4. Spin at max speed (21130 xg) for 5 minutes at 15°C
5. Transfer 9 µl supernatant to MS sample vials and add 1 µl iRT peptides

6. MS Measurement

1. The samples are injected on an Orbitrap Fusion Lumos Tribrid mass spectrometer equipped with an Aquity M-Class UPLC System
2. Peptides are separated on a self-pack 40 cm x 0.75 µm i.d. column packed with 1.9 µm C18 beads using a linear gradient of 3% to 35% acetonitrile in water with 0.1% FA over the course of 120 minutes at flow rate of 300 nl/min
3. The MS1 spectra are acquired in a scan range of 350-1500 m/z with an Orbitrap resolution of 120000 with the normalized AGC target set to 200% with a maximum injection time of 264 ms and the RF lens was to 50%
4. The targeted MS2 spectra are acquired for the desired masses with variable isolation windows listed below and fragmented with a HCD of 30%
    a. The spectra are measured with an Orbitrap resolution of 30000 with variable scan ranges and the AGC target set to 200% with a maximum injection time of 54 ms and the RF lens set to 50%

Window	Lower bound (m/z)	Upper bound (m/z)	Width (m/z)
1	349.5	381.5	32
2	380.5	408.5	28
3	407.5	432.5	25
4	431.5	458.5	27
5	457.5	482.5	25
6	481.5	506.5	25
7	505.5	530.5	25
8	529.5	553.5	24
9	552.5	578.5	26
10	577.5	605.5	28

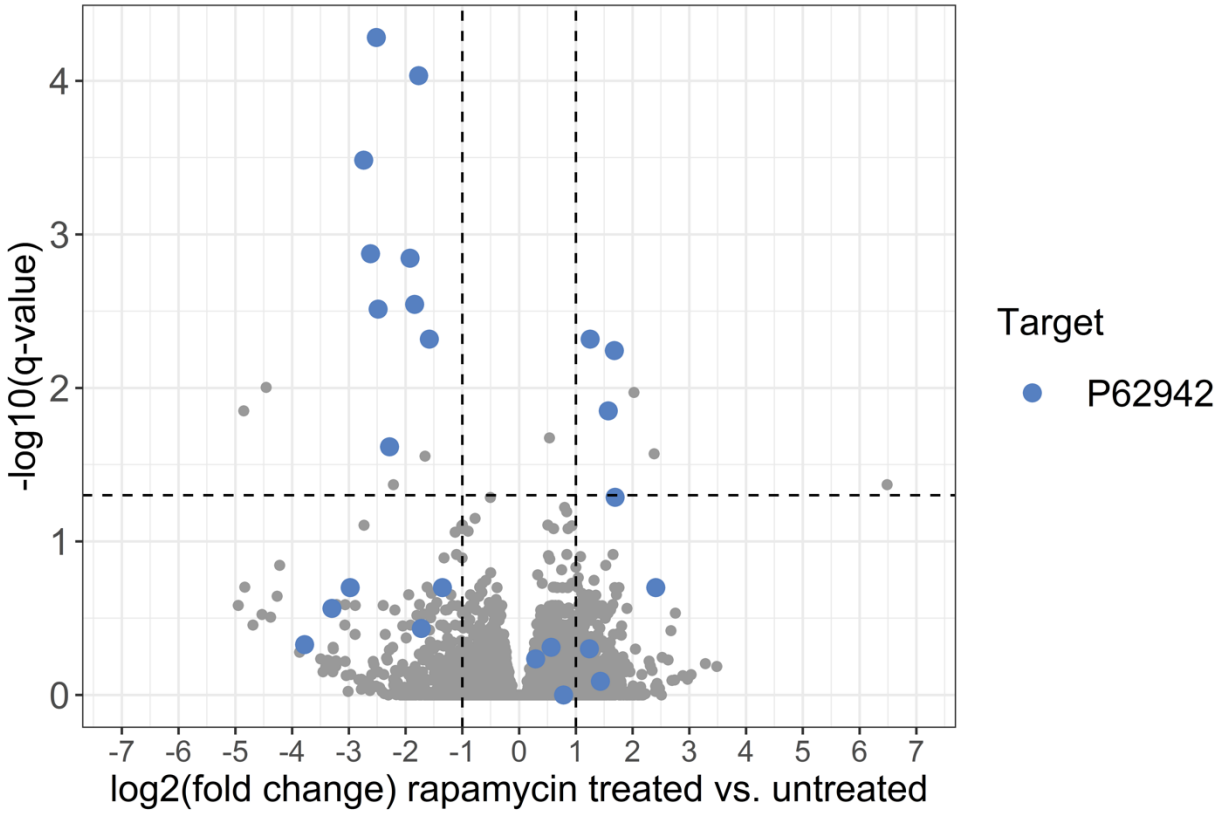
11	604.5	633.5	29
12	632.5	662.5	30
13	661.5	693.5	32
14	692.5	727.5	35
15	726.5	766.5	40
16	765.5	809.5	44
17	808.5	862.5	54
18	861.5	929.5	68
19	928.5	1029.5	101
20	1028.5	1498.5	470

7. Data analysis

1. The *directDIA* feature on Spectronaut version 15 is used for identification and quantification of the DIA data
    a. We use modified BGS factory settings as all precursors are considered individually to allow interpretation of peptide level data
2. The data are exported from Spectronaut and further analyzed in Rstudio using R 4.0.2
3. We use the R package protti for data normalization, filtering, quality control, differential abundance calculation, and significance testing
    a. See Jan-Philipp Quast, Dina Schuster, Paola Picotti, protti: an R package for comprehensive data analysis of peptide- and protein-centric bottom-up proteomics data, *Bioinformatics Advances*, Volume 2, Issue 1, 2022, vbab041, https://doi.org/10.1093/bioadv/vbab041

8. Anticipated results

If applied for the first time we recommend to include 10 µM rapamycin treatment as a positive control. In a successful LiP-MS experiment, this should result in significant abundance changes of peptides derived from the rapamycin target FKBP12 (P62942) as shown in the volcano plot below:


-log10(q-value)
4
3
2
1
0
-7
-6
-5
-4
-3
-2
-1
0
1
2
3
4
5
6
7
log2(fold change) rapamycin treated vs. untreated
Target
P62942