Sample preparation guidelines for chemical cross-linking mass spectrometry (XL-MS)

We have assembled the following guidelines for the analysis of protein complexes by XL-MS. In case you have further questions please do not hesitate to contact us.

1) Purity of Proteins:

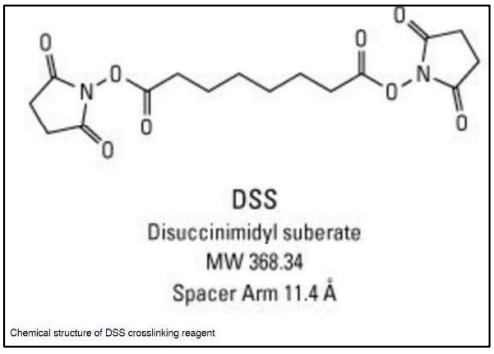
The proteins targeted for cross-linking should be of **high purity**. The analysis is easier with recombinantly expressed proteins and with complexes containing a low number of proteins (up to 5) than with complexes purified by immunoprecipitation. For higher complex samples, an additional enrichment step of the cross-linked peptides may be required.

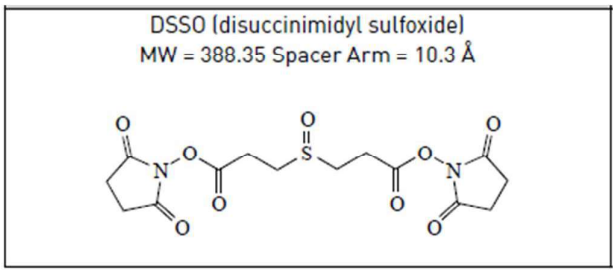
2) Amount of Protein

Required **amount of protein**: appr. 10 µg / reaction (a band visible on a Coomassie gel is usually enough). To prove that your proteins of interest form a complex a native PAGE can be performed.

3) Cross-linking Chemistry

Which **cross-linking chemistry** will be applied? There are numerous cross-linkers available with different chemistries, advantages/disadvantages and sometimes also special requirements.
In general, we will apply one of the most widely used cross-linker type, which is an amine reactive cross-linker (NH2 - NH2). There are several reagents available, such as BS3 and DSS (11.4 Å), BS2G (7.7 Å), DSSO (10.3 Å)





4) Choose cross-linking compatible buffer and pH:

For amine-reactive cross-linking choose a non-amine containing buffer at pH 7-9 (e.g. 20 mM HEPES pH 7.8). Please avoid TRIS, ammonium bicarbonate or Glycine buffer.

IMPORTANT: please note that many cross-linkers are very unstable, especially in water, and thus should be treated very carefully. You can get aliquots of different cross-linkers in the Protein Chemistry facility. For BS3, DSS and DSSO we recommend dissolving them in water-free DMSO directly before use.

In case, that the cross-linked proteins will be directly digested and analyzed in solution (without a separation by SDS-PAGE) the cross-linking reaction has to be performed in MS-compatible buffers, i.e. free of detergents (Triton, NP40,..) and protease inhibitors.

5) Optimize cross-linking reaction

The target protein concentration should be in the range of 10-20 µM and not higher than 0.5-5mg/ml. The cross-linker concentration differs depending on the cross-linking chemistry. For BS3, DSS, DSSO: use about 5- to 50-fold molar excess over the protein concentration

The optimum concentrations have to be worked out for each sample:

- Do a cross-linker titration and check on SDS PAGE to find the ideal concentration
- Vary the concentrations of both or one of your interacting proteins to promote complex formation.
- Using too high concentrations of protein and cross-linker will result in the formation of unspecific complexes and aggregates
- Check out the websites of Thermo Fisher and creative molecules for further useful information:

https://www.thermofisher.com/at/en/home/life-science/protein-biology/protein-labeling-crosslinking/protein-crosslinking.html
http://www.creativemolecules.com/CM_Products.htm

6) Sample submission

- Please always contact us before submitting samples.
- Samples can be submitted in-gel or in-solution. The standard procedure would be to digest them in solution, if most of the protein is in a complex and no unspecific reactions and aggregates are visible on the test gel. We can also cut the band(s) of the cross-linked proteins and digest them in-gel.
- Please consider that sometimes monomer bands also contain important information when compared to a di- or multimer because they allow differentiating intra- from intermolecular cross-links.
- Please attach a scan/picture of the gel and accession numbers of your proteins to the submission form. If the proteins are tagged constructs, please also attach the complete amino acid sequences.

7) The work we will provide for XL-MS

If you need any advice on planning or optimizing your cross-linking experiment, contact Elisabeth Roitinger. We will perform an in-gel or in-solution digest using trypsin or if required alternative enzymes and analyse the sample by nano-LC-MS/MS with or without prefractionation by Size exclusion chromatography.

As a result, we will provide a list of identified proteins and cross-links (pLink database search for DSS, BS3 and XlinkX search or MS Annika search for DSSO).

Please bear in mind that there might be many reasons why sometimes no or only few cross-links are identified:

1. the cross-linked peptides are too short or too long for analysis or reliable identification,
2. the fragmentation of the cross-linked peptide is not ideal and hampers identification,
3. the cross-linked peptide is below the detection threshold,
4. the cross-linker is too short to link suitable residues,
5. the primary amino acid sequence is not well suited for the cross-linker chemistry with respect to reactive (XL) or cleavable (digest) residues,
6. the reaction conditions were not ideal,
7. the overall amount is too low,
8. the cross-linked peptides are difficult to extract from gel (in-solution digest as alternative) – to name the most common reasons.

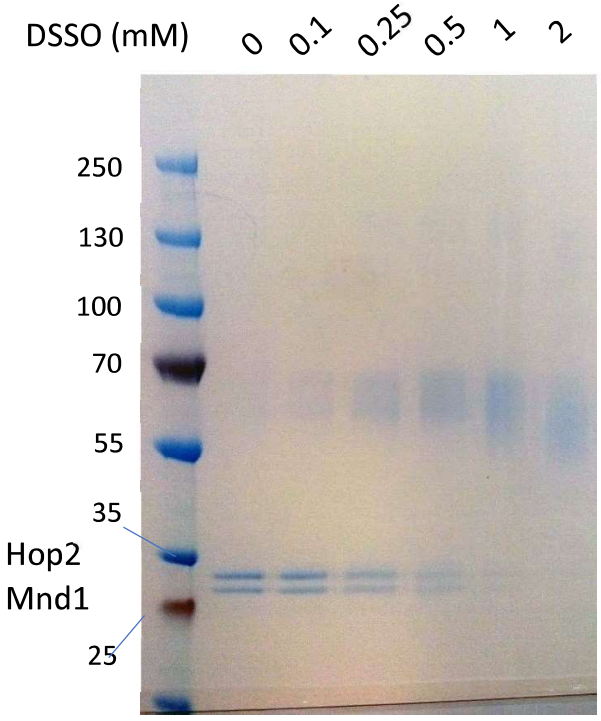
Example of an optimization experiment:

The A.th proteins Hop2 and Mnd1 were cross-linked with DSSO at different protein to cross-linker ratios.

Protein concentration: 20 μM (0.5 mg/ml of each protein)
Cross-linker concentrations: 0.1 to 2 mM
Buffer: 50 mM phosphate buffer pH 8.0, 25 mM NaCl

Procedure:

- *Hop2 and Mnd1 were cross-linked using DSSO at different protein to cross-linker ratios for 30min at 37C*
- *The cross-linking reaction was stopped by adding Ammonium bicarbonate to a final concentration of 50mM*
- *About 0.4 ug of each Xl reaction was analyzed by SDS-PAGE*



Acknowledgement: These guidelines were assembled based on the guidelines provided by the MFPL mass spectrometry facility