Sample preparation guidelines for MS-based cross-linking (XL-MS)

We have assembled the following guidelines to help you designing your cross-linking experiment. In case you have further questions please do not hesitate to contact us.

1) The proteins targeted for cross-linking have to be of **high purity**. Simple IPs from cell lysates usually yield far too much background and cannot be used in a standard XL-MS setup.

2) Required **amount of protein**: a few µg (a band visible on a Coomassie gel is usually enough)

3) Which **cross-linking chemistry** is best for your complex?
There are numerous cross-linkers available with different chemistries, advantages/disadvantages and sometimes also special requirements. Consider, that the protein sequence of interest will determine which combination of crosslinker and cleavage-enzyme yields best results. In general, we recommend to start with one of the most widely used cross-linker types (see also 7e):

a. Amine reactive cross-linking (NH2 - NH2): e.g. BS3 and DSS (11.4 Å), BS2G (7.7 Å)
b. Carboxyl-amine cross-linking (COOH - NH2): e.g. EDC/sulfo-NHS (zero-length)

4) Choose **cross-linking compatible buffer and pH**:

a. Amine-reactive cross-linking: non-amine containing buffer at pH 7-9 (e.g. 20 mM HEPES pH 7.8)
b. Carboxyl-amine cross-linking: non-amine, non-carboxylate buffer at pH 4.5 -7.2 (e.g. 0.1 M MES buffer pH 6.5)
c. **IMPORTANT**: please note that many cross-linkers are very unstable, especially in water, and thus should be treated very carefully. For BS3 and DSS we recommend preparing stock solutions in water-free DMSO and storing small aliquots at -80°C. Thaw aliquots directly before use.

5) Optimize cross-linking reaction

a. Target protein concentration should be in the range of 10-20 µM
b. Cross-linker concentration differs depending on cross-linking chemistry
    i. BS3, DSS: about 5- to 50-fold molar excess of the protein concentration
    ii. EDC/sulfo-NHS: EDC about 2 mM and sulfo-NHS about 5 mM (EDC to sulfo-NHS ratio of 1:2.5)
c. The optimum concentrations will depend on several factors:
    - The above mentioned protein and cross-linker concentrations have been proven to be effective in our cross-linking experiments, but these parameters have to be adjusted and optimized for each complex.
    - 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 and Creative Molecules Inc. for further useful information:

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

6) Sample submission

a. Please always contact us before submitting samples.
b. Samples can be submitted in-gel or in-solution. The standard procedure would be to cut the band(s) of the cross-linked proteins and digest them in-gel. However, if most of the protein is in complex and no unspecific reactions and aggregates are visible on the gel, we could also digest the sample in-solution.
c. 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.
d. Please attach a scan/picture of the gel and accession numbers of your proteins to the submission form. If the proteins are constructs, please also attach the complete amino acid sequences.

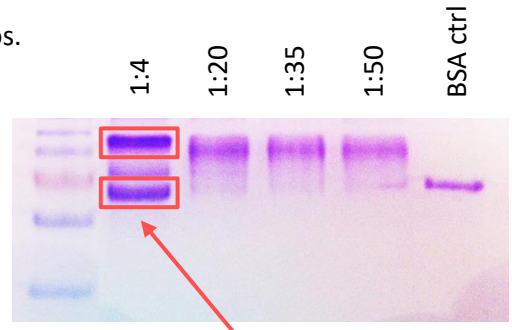
7) The service we will provide for XL-MS

a. If you need any advice on planning or optimizing your cross-linking experiment, feel free to contact us (proteomics@mfpl.ac.at).
b. We perform tryptic in-gel or in-solution digestion, alternative enzymes upon request.
c. Samples will be analyzed using a nano-LC-MS/MS setup with a 2h gradient.
d. We will provide following results:
    i. List of identified proteins (MaxQuant database search)
    ii. List of identified cross-links (pLink database search)
e. Please bear in mind that there might be a number of 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.
f. Further services like manual validation of identified cross-linked peptides only upon request.

Examples of optimization experiments:

1) Amine-reactive cross-linking

BSA crosslinked using BS3 at different protein to cross-linker ratios.



Protein concentration: 15 µM (1mg/ml)
Cross-linker concentrations: 60, 300, 525, 750 µM BS3
Buffer: 20 mM HEPES-KOH, 20 mM NaCl, 5 mM MgCl2, pH 7.8

Procedure:

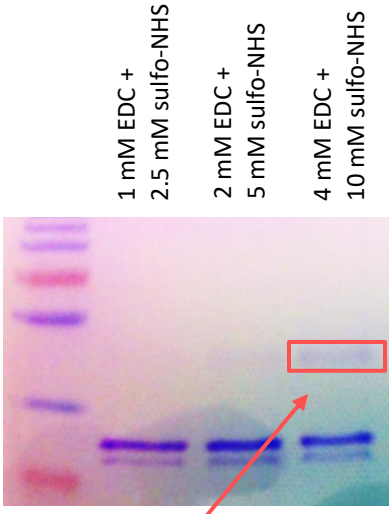
- BSA was crosslinked using BS3 at different protein to cross-linker ratios for 1 hour at room temperature in the dark.
- The crosslinking reaction was stopped by adding Tris pH 7.8 to a final concentration of 50 mM.
- About 5 µg of each cross-linking reaction was loaded on the SDS PAGE

Monomer and dimer bands of crosslinked BSA were used for further analysis, because we were interested in intramolecular and intermolecular crosslinks.

2) Carboxyl-amine cross-linking

Heterodimer crosslinked using EDC/sulfo-NHS

Protein concentration: 5 µM each protein = 10 µM
Crosslinker concentrations: 1, 2, 4 mM EDC + 2.5, 5, 10 mM sulfo-NHS
Buffer: 0.1 M MES-NaOH ph 6.5, 0.5 M NaCl, 2 mM CaCl2



Procedure (one-step protocol):

- The proteins were crosslinked using EDC and sulfo-NHS at different protein to crosslinker ratios and incubated for 30 min at RT in the dark
- 20mM β-mercaptoethanol was added to quench excessive EDC and the amine coupling was stopped by adding Tris pH 7.8 to a final concentration of 50 mM.
- About 2 µg of each cross-linking reaction was loaded on the SDS PAGE

Band of crosslinked complex was used for further analysis. This represents about the minimum amount that is necessary. Stronger bands for the cross-linked complex are highly desirable, as long as no over-crosslinking is observed.