A composite filter for low FDR of protein-protein interactions detected by in vivo cross-linking

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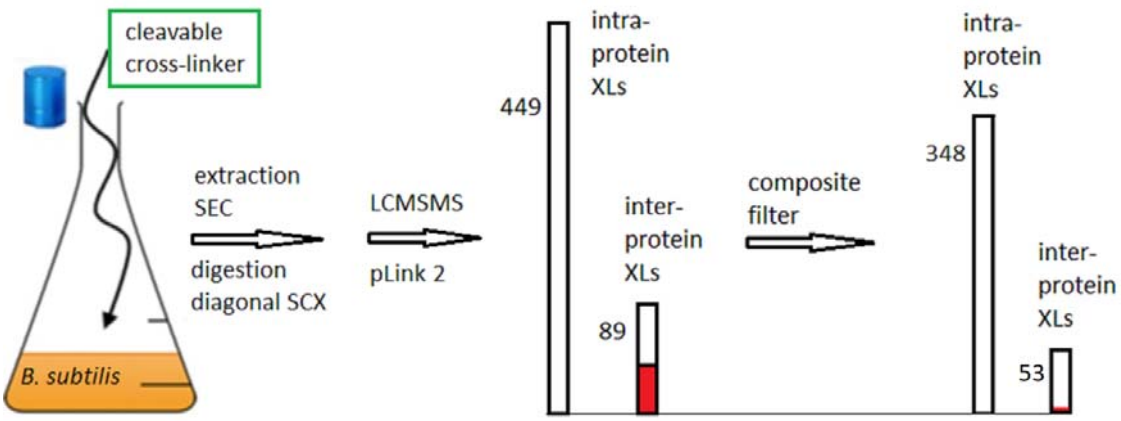
Abstract

In vivo chemical cross-linking combined with LCMSMS of digested extracts (in vivo CX-MS) can reveal stable and dynamic protein-protein interactions at a proteome wide-scale and at the peptide level. In vivo CX-MS requires a membrane permeable and cleavable cross-linker that enables isolation of target peptides and a fast and sensitive search engine to identify the linked peptides. Here we explore the use of the search engine pLink 2 for analysis of a previously obtained LCMSMS dataset from exponentially growing *Bacillus subtilis* treated in culture with the cross-linker bis(succinimidyl)-3-azidomethyl-glutarate (BAMG). Cross-linked peptide pairs were identified by pLink 2 in very short time at an overall FDR of < 5%. To also obtain a FDR < 5% for inter-protein cross-linked peptide pairs additional thresholds values were applied for matched fragment intensity and for the numbers of unambiguous y and b ions to be assigned for both composite peptides. Threshold values were based on a set of decoy sequences from yeast and human sequence databases. Also the mass- and charge-dependent retention times of target peptides purified by diagonal strong cation exchange chromatography were used as a criterion to distinguish true from false positives. After this filtering, pLink 2 identified more than 80% of previously reported protein-protein interactions. In addition the use of pLink 2 revealed interesting new inter-protein cross-linked peptide pairs, among others showing interactions between the global transcriptional repressor AbrB and elongation factor Tu and between the essential protein YlaN of unknown function and the ferric uptake repressor Fur.

Keywords

Cross-linking mass spectrometry, cleavable cross-linker, protein-protein interactions, YlaN, Fur



Highlights

- Improved protocol for identification of PPIs at low FDR by in vivo cross-linking with BAMG
- The use of all intra-protein cross-linked peptide pairs as true positives
- The cytosolic aminopeptidase (AMPA_BACSU) interacts with the 50S ribosomal protein L17
- The transition state regulator AbrB interacts with elongation factor Tu
- The essential protein YlaN of unknown function interacts with the iron uptake repressor Fur

Significance

Important for reliable identification of PPIs by chemical cross-linking in vivo is a low FDR of non-redundant inter-protein peptide pairs. Here we describe how to recognize the presence of spurious interactions in a dataset of cross-linked peptide pairs enriched by 2D strong cation exchange chromatography and identified by LCMSMS by taking into account chromatographic behavior of cross-linked peptide pairs and protein abundance of corresponding peptides. Based on these criteria we assessed that the FDR of the fraction of non-redundant inter-protein cross-linked peptide pairs was approx. 20-25% by interrogating an entire species specific database at an overall FDR of 5% or 0.1% with a search engine that otherwise scores best in sensitivity among other search engines. We have defined a composite filter to decrease this high FDR of inter-protein cross-linked peptide pairs to only about 2%.

1. Introduction

Specific protein-protein interactions (PPIs) are crucial for the regulation of biochemical processes. Large scale approaches like affinity purification combined with mass spectrometry[1], proximity-dependent labeling[2], the use of co-elution profiles[3] and the yeast two-hybrid system[4] can reveal which proteins interact with each other under different experimental and physiological conditions. In vivo chemical cross-linking followed by mass spectrometry and database searching to identify cross-linked peptide pairs (CX-MS) has the potency to reveal cellular protein-protein interactions at a proteome-wide scale in a single experiment and in a short time[5–7]. Besides stable protein complexes also dynamic assemblies that may dissociate upon cell extraction can be trapped by cross-linking in vivo, implying that PPIs may be encountered that have thus far escaped detection by in vitro approaches. Most importantly, the spatial arrangement of proteins in a complex can be assessed by CX-MS by virtue of the identity of linked amino acid residues in the protein sequences as determined by MS and the known length of the spacer of the cross-link. The presence of cross-links formed between two proteins in a complex facilitates modeling of the overall structure if the coordinates of the atoms in the two interacting proteins are known. Such models may lead to hypotheses about the functional significance of the interaction. Thus, CX-MS in vivo is a useful approach in structural and systems biology.

In vivo CX-MS requires a membrane permeable cross-linker designed to facilitate mass spectrometric identification of cross-linked sites. Peptide identification is achieved by liquid chromatography coupled to mass spectrometry (LCMSMS). Mass and charge of the peptides are determined in the MS1 stage. In the MS2 stage peptides are selected for fragmentation by cleavages of the peptide bonds induced by collision with gas molecules (collision-induced dissociation, CID) in a data dependent way, i.e., selection is dependent on signal intensity, mass and charge, and whether a particular precursor ion has been selected before in a given time window. This results in an MS2

26 fragment spectrum that is characteristic for the peptide sequence. With an MS1MS2 dataset,
27 unmodified peptides, or peptides with a defined modification, can be identified by searching in a
28 sequence database. However, for cross-linked peptides this approach is challenging for two main
29 reasons. In the first place the MS1 data give no information about the masses of the composite
30 peptides, which hampers their identification by database searching. The lack of peptide mass
31 knowledge can be circumvented using a gas phase cleavable cross-linker yielding fragment ions from
32 which the masses of the composite peptides can be deduced. In the second place cross-linked
33 peptides are present in sub-stoichiometric amounts as compared with unmodified peptides, so that
34 selection in the mass spectrometer in the process of data dependent acquisition of MS1MS2 spectra
35 is hampered. This problem requires enrichment of the rare cross-linked species out of the bulk of
36 unmodified peptides.

37 To meet these requirements and challenges we previously synthesized bis(succinimidyl)-3-
38 azidomethyl-glutarate (BAMG)[8], a membrane permeable cross-linker[9]. The short spacer of BAMG
39 gives relatively high resolution cross-link maps, while the azido group in the spacer can be modified
40 in different ways. This reactive versatility enables three different cross-link analysis strategies[10–
41 14]. For CX-MS in vivo we have made use of a TCEP-induced reduction of the azido group to enrich
42 cross-linked peptide pairs obtained from growing *Bacillus subtilis* cells treated in culture with
43 BAMG[9]. Besides isolation of target peptides, reduction of the azido group also facilitates mass
44 spectrometric identification of the linked peptide pair. This is due to the fact that the two cross-link
45 amide bonds of a peptide pair are cleavable in the gas phase by CID [11]. The cleavage of the cross-
46 link amide bond can occur along with a peptide bond cleavage. The principle was demonstrated
47 with protein complexes from *Bacillus subtilis* in the mass range 400 kDa to 1-2 MDa obtained by size
48 exclusion chromatography (SEC) after in vivo cross-linking. We identified several cross-linked
49 peptides revealing transient and stable PPIs of high biological significance and low FDR by searching
50 MS1MS2 data from the entire *B. subtilis* sequence database[9]. This analysis was supported by the

51 use of in-house developed scripts called Raeng and YeunYan in combination with the search engine
52 MASCOT.

53 This procedure, further called the Raeng/Mascot approach, is relatively laborious, making it
54 unattractive for general use. This holds in particular in cases were large datasets are to be expected
55 by the use of state of the art HPLC coupled to sensitive and fast mass spectrometers like the recently
56 introduced trapped ion mobility time-of flight mass spectrometer (timsTOF Pro) enabling online
57 parallel accumulation-serial fragmentation (PASEF)[15]. Here we explore the use of the recently
58 launched second generation search engine, pLink 2[16]. In comparison with many other search
59 engines developed for cross-link analysis, pLink performs best with respect to number of identified
60 cross-links with good FDR at cross-link spectrum matches (CSM) level [16,17]. Moreover it is
61 extremely fast, and is also suitable for analysis using cleavable cross-linkers like BAMG[16]. With an
62 existing LCMSMS dataset we observed a large overlap with the results obtained with the
63 Raeng/Mascot approach, but also found evidence that the FDR for inter-protein peptide pairs is
64 higher for inter-protein peptide pairs than for intra-protein peptide pairs. Here we describe an
65 approach to further lower the FDR for the latter type of cross-links that reliably led to interesting
66 new protein-protein interactions.

67

68 **2. Materials and methods.**

69 *2.1. Acquisition of the LCMSMS dataset to explore the use of pLink 2.*

70 The LCMSMS dataset was acquired as described previously in detail [9]. In short, exponentially
71 growing *B. subtilis* (strain 168) was treated in culture with 2 mM BAMG at 37° C for 5 min. The
72 soluble extract, obtained after harvest and sonication of cross-linker-treated cells, was subjected to
73 SEC. A tryptic digest of protein complexes in the size rage 400 kDa to 1-2 MDa was selected for
74 further analysis. First the peptide mixture was subjected to diagonal strong cation exchange (SCX)

75 chromatography to sequester the cross-linked peptides from the bulk of unmodified peptides.
76 Fractions enriched in cross-linked peptide pairs were analyzed by LCMSMS using an Eksigent Expert
77 nanoLC 425 system connected to the Nano spray source of a TripleTOF 5600+ mass spectrometer.
78 Data processing was as described [9]. For analysis by pLink 2 the original mgf files were combined
79 into 13 mgf files. An overview of the origin and size of the combined mgf files along with the mass
80 accuracy used for pLink 2 searches is shown in Supplementary Table S1. The original mgf files are
81 available via ProteomeXchange with identifier PXD006287.

82 *2.2. Identification of cross-linked peptides*

83 BAMG-cross-linked peptides become cleavable in the gas phase when the azido group in the spacer
84 of the cross-link has been reduced to an amino group. For cross-link identification we used pLink 2 as
85 a search engine operating in the stepped-HCD mode for MS-cleavable cross-linkers [16]. This mode
86 has been developed for the cross-linkers DSBU[18] and DSSO[19]. In the stepped-HCD mode
87 cleavage of the DSSO- or DSBU-cross-link and generation of peptide fragments occurs with two
88 different CID energies after which the data obtained in the two steps are combined in one file entry.
89 Cleavage of the BAMG-cross-link amide bonds and cleavage of the peptide bonds resulting in y and b
90 ions occurs in one step under defined CID conditions (Fig 1). Therefore both DSSO-, DSBU- and
91 BAMG-cross-linked α-β peptide pairs yield similar MSMS spectra containing the signals from intact
92 cleaved peptides along with the signals from peptide bond cleavages. Like DSSO and DSBU, BAMG is
93 scissile at two identical sites. This implies that a single cleavage event in the members of an
94 ensemble of identical cross-linked peptide pairs results in a mixture of two pairs of products with a
95 characteristic mass difference. The short versions of the cleaved peptides are denoted αS and βS and
96 the long version are denoted αL and βL. For BAMG the mass difference between αS and αL and
97 between βS and βL is 125.048 Da, αL and βL being modified by the remnant of the cross-linker in the
98 form of a γ-lactam, while αS and βS are unmodified after the cleavage (Fig 1). The presence of four
99 such cleavage products directly reveals the masses of the composite peptides. Also double cleavage

100 events usually take place, by which cleavage of a cross-link amide bond occurs along with a peptide
101 bond cleavage. Often signals from only one pair of cleavage products are detectable, usually from
102 the shortest composite peptide, the other pair of cleavage products being completely fragmented by
103 secondary cleavages. If only one pair of peaks with a 125 Da mass difference is present in the mass
104 spectrum, the mass of the other peptide can be calculated by subtracting the mass of the first
105 peptide from the mass of the precursor ion. We have shown that 83% of the mass spectra from 401
106 different cross-linked peptide pairs display at least one pair of cleavage products from either the α-
107 peptide or the β-peptide. In principle the presence of 2 cleavage products in the combinations αS
108 and βS, αS and βL, αL and βS and αL and βL can also be used to deduce the masses of α and β. These
109 combinations occur in an additional 5% of the instances [11]. Peptide bond cleavages can also result
110 in ion pairs differing 125.048 Da, denoted αSy or αSb for the short and αLy or αLb for the long (+
111 125.048 Da) versions of y or b ions from the α peptide. For the β peptide short and long version of y
112 and b ions are denoted βSy, βLy, βSb and βLb. pLink 2 was adapted for BAMG cross-link
113 identification by considering BAMG-specific fragment ions. It is anticipated that the presence of y-
114 ion or b-ion pairs with mass differences of 125.024 Da will not prevent assessment of the correct
115 masses of α and β by pLink 2, although the presence of these ion pairs may result in the calculation
116 of more than one candidate for the masses of α and β of a given precursor ion.

117 Four different protein databases were used for identification; (i) the Uniprot protein database of
118 *Bacillus subtilis* (4260 entries); (ii) a hybrid database composed of the proteins from database (i) and
119 the Uniprot protein database of *Saccharomyces cerevisiae* (6043 entries) [20]; (iii) a database of the
120 673 proteins identified in the primary SCX fractions and (iv) a hybrid database composed of the
121 proteins from database (iii) and 1085 human proteins identified in a SEC fraction of a HeLa cell
122 nuclear extract[12]. The yeast and human proteins in the hybrid databases are used as a source of
123 decoy sequences for FDR estimations.

124 *2.3. Mass spectrometric criteria for cross-link identity assignment*

125 The following parameters were used for pLink 2 searches of tryptic peptides using the mgf dataset as
126 shown in Supplementary Table S1; mass accuracy for precursor and fragment ions: 25-75 ppm,
127 depending on the mgf file; up to two missed cleavages allowed; masses: 600-6000; lengths for α and
128 β peptides: 6-60 amino acids; a carbamidomethyl group at C as fixed modification; oxidation at M as
129 variable modification; 5% or 0.1% FDR for cross-linked spectrum matches. With these settings intra-
130 protein cross-links were assigned as nominated by pLink 2. For assignment of inter-protein cross-
131 links the following additional requirements were taken into account: the assignment of least 4
132 unambiguous y ions for α and β peptides with a length of 12 amino acids or more, and at least 3
133 unambiguous y ions for peptides consisting of 11 amino acids or less along with a matched intensity
134 scoring higher than 35%. For a matched intensity score of more than 50%, at least 2 unambiguous y
135 ions are sufficient for assignment of peptides consisting of 11 amino acids or less, provided that also
136 at least 2 unambiguous b ions can be assigned to the peptide. A y or b ion is considered ambiguous if
137 it can also be assigned to one or more other fragments. A yS and yL or bS and bL ion pair with the
138 125 Da mass difference is counted only once for the requirement with respect to the minimal
139 number of unambiguous y or b ions for validation and assignment. Doubly charged y or b ions at m/z
140 ≤ 700 are not taken into account. The ignorance of b ions as a selection criterion, except for short
141 peptides and at high matched intensity as described above, is based on their relatively low
142 occurrence as compared with y ions [9].

143 Matched intensity and numbers of unambiguously assigned y and b ions are mentioned in result
144 tables only once for candidates of which more than one MSMS result was put forward as a result of
145 multiple selections for MSMS by the mass spectrometer. Usually the MSMS spectrum with the
146 highest matched intensity was chosen, provided that also the other criteria for assignment were
147 met.

148 *2.3. Elution time window during SCX as a criterion for cross-link identity assignment*

149 A further filter concerned the mass of a cross-linked peptide pair and the calculated charge state in
150 relation to the elution time. The charge state is calculated at pH 3, i.e., the conditions of strong
151 cation exchange chromatography, assuming protonation of all acid and basic groups. To asses the
152 distribution of mass and charge in relation of elution time in SCX chromatography of true positives
153 we took into account all intra-protein cross-linked peptide pairs identified by pLink 2 and only the
154 inter-protein peptide pairs that were identified by both Raeng and pLink 2. Identical peptide pairs
155 that eluted in different SCX fractions were also taken into account.

156 *2.4. FDR estimation; the use of different decoy sequences for inter- and intra-protein peptide pairs;*
157 *selectivity and sensitivity*

158 The overall FDR is defined as FDR = d/(d + t) x 100%, in which d is the number of decoy α-β sequence
159 hits and t is the number of identified target α-β sequences. A decoy α-β peptide pair consists of two
160 human or yeast sequences or of one human or yeast and one target (*B. subtilis*) sequence. For intra-
161 protein cross-linked peptide pairs, decoy sequences for α and β are from the same yeast or human
162 protein, whereas for inter-protein peptide pairs the decoy sequences are from different proteins.
163 Also target-reversed and reversed-reversed peptide pairs with different sequences of α and β
164 peptides from the same proteins are decoy sequences for intra-protein cross-linked peptide pairs.
165 However, previously we showed that no reversed-reversed versions and only three target-reversed
166 versions of these intra-protein decoy sequences were put forward on a total of 1288 decoy hits[11].

167 *3.* **Results and discussion**

168 *3.1. Protein composition of the extract obtained after in vivo cross-linking of exponentially growing*
169 *cells with BAMG*

170 Previously we have identified cross-linked peptide pairs from protein complexes in the size range
171 400 kDa to 1-2 MDa after SEC of an extract obtained from an exponentially growing *B. subtilis*
172 culture treated with BAMG. A size exclusion chromatogram of the protein extract is shown in Fig 2.

173 In the selected SEC fractions 4-7 we identified 673 proteins [9]. The known MW of most of these
174 proteins is much smaller that the size range of 400 kD to 1-2 MDa. Besides specific cross-linking in
175 stable and transient protein complexes also cross-linking during random diffusional encounters
176 between proteins in the concentrated cytosol may attribute to this high molecular weight shift and
177 size heterogeneity. It is anticipated that only specific interactions will be found, assuming that in a
178 digest the many particular non-specific cross-links are so rare that they will remain largely
179 undetected by LCMSMS.

180 *3.2. Isolation of cross-linked peptides by diagonal strong cation exchange (SCX) chromatography*

181 The principle of diagonal chromatography to isolate BAMG-cross-linked peptides from the bulk of
182 unmodified peptides [12] is depicted in Fig 3. Isolation of peptides with a specific reactivity by two-
183 dimensional liquid chromatography was introduced in 2002 in order to increase the numbers of
184 proteins identified by LCMSMS [21]. In this approach, coined diagonal chromatography, target
185 peptides in chromatographic fractions are subjected to a reaction that modifies their
186 chromatographic retention time. In the second dimension peptides in treated fractions sequester
187 from the bulk of unmodified peptides that elute at the same retention time as in the primary run.
188 Here we use reduction of the azido group to an amino group on the spacer of a crosslink in peptides
189 to increase the charge state. Also peptides with an internal cross-link, called loop-linked peptides
190 and peptides with one modified lysine residue, called mono-linked peptides, the second reactive
191 ester of the cross-linker being hydrolyzed, are enriched by diagonal SCX chromatography.

192 An additional advantage of purification of target peptides by diagonal SCX chromatography is the
193 characteristic relationship between de elution time and the mass range and positive charge range of
194 cross-linked peptide pairs at pH 3.0 of the mobile phase (Table 1). Table 1 has been composed from
195 the data of 626 cross-linked peptide pairs (section 2.4), showing that (i) highly charged cross-linked
196 peptide pairs tend to elute late and (ii) at each charge state 4^+, 5^+ and 6^+ the mass range decreases at
197 increasing elution time. Three 5^+ charged peptides from fraction 10 and one 6^+ charge peptide from

198 fraction 12 form the only exceptions to the otherwise consistent distribution. This consistency
199 enables the use of elution time in relation to the charge and mass of a candidate cross-link as one of
200 the criteria to discriminate between true and false positives.

201 *3.3. pLink 2 is a fast search engine for identification of BAMG-cross-linked peptides, but for a FDR*
202 *< 5% for inter-protein cross-linked peptide pairs further filtering is required.* We tested the suitability
203 of pLink 2 as a search engine to identify BAMG-cross-linked peptides using an existing LCMSMS
204 dataset. The dataset consisted of mgf files from LCMSMS analysis of 10 fractions obtained by
205 diagonal SCX chromatography of trypsin-digested material present in SEC fraction 4-7. Previously we
206 identified with the Raeng/Mascot approach several inter-protein cross-linked peptide pairs that
207 fulfill the requirements of a minimal peptide length of 6 amino acids for identification by pLink 2
208 (Table 2). These inter-protein cross-linked peptide pairs revealed 41 interactions between different
209 proteins. The dataset also contained several homo-dimeric peptide pairs and intra-protein peptide
210 pairs. Although the results were obtained by searching the entire *B. subtilis* sequence database, all
211 inter-protein cross-linked peptide pairs belong to the set of 673 actually identified proteins in the
212 analyzed SEC material. Only a few homo-dimeric and intra-protein peptide pairs did not belong to
213 the 673 identified proteins.

214 The 13 MSMS data files (supplementary Table S1) in mgf format with an average size of about 14.4
215 MB were separately analyzed by pLink in the very short time of about 3 min on average per mgf file.
216 Nearly 10,000 spectra were identified by pLink 2 at less than 5% FDR (Table 3), distributed over intra-
217 protein and inter-protein cross-linked peptide pairs, mono-linked peptides, loop-linked peptides and
218 regular peptides. Table 2 depicts the numbers of the non-redundant intra-protein cross-linked
219 peptide pairs, inter-protein peptide pairs and homo-dimeric peptide-pairs identified by pLink 2. Less
220 than 3% of the intra-protein cross-linked peptide pairs did not belong to the 673 independently
221 identified proteins (Supplementary Table S2, column F, salmon-pink highlighted). However, no less
222 than 24 out of the 89 inter-protein cross-linked peptide pairs nominated by plink 2 did not belong to

223 the 673 identified proteins (Supplementary Table S2, column F, pink and cyan highlighted).
224 Moreover, 16 of the 89 nominated species violated the expected elution time in CX chromatography
225 based on their mass and calculated charge at the pH (pH 3.0) of the mobile phase in SCX
226 chromatography (Table 1 and Supplementary Table S2, column D, blue highlighted). The presence of
227 a relatively large number of inter-protein peptide pair candidates not belonging to the 673 most
228 abundant proteins and the violation by several inter-protein peptide pairs of the mass and charge
229 rules of the SCX elution times strongly suggests the presence of false positive nominations among
230 these inter-protein cross-linked species. This observation is not unexpected, since it is well
231 documented that by searching a large sequence database at a given FDR at CSM level, practically all
232 false positives are confined to inter-protein peptide pairs [9–11]. Based on a total of 1856 spectra of
233 cross-linked peptide pairs (Table 3) we roughly calculate a FDR at CSM level of 1.7% assuming that
234 the 32 total inter-peptide cross-linked peptide pairs with aberrant SCX elution time, or of which at
235 least one peptide does not belong to the 673 independently proteins, are false positives. This would
236 imply a FDR of 11.3% for the 252 inter-protein peptide pairs or, with 30 non-redundant decoy hits,
237 about 25% for the 89 non-redundant inter-protein peptide pairs put forward by pLink 2 (Tabel 2).
238 The FDR would be even larger if related to PPIs. These trends are in agreement with a previous
239 discussion on FDR estimations on different levels[22].

240 *3.4. A target-decoy database to find criteria for a low FDR of inter-protein peptide pairs*

241 A target-decoy database is used by pLink 2 for FDR calculation. The decoy database consists of the
242 reversed sequence of the target database. Since reversed sequences are not reported by pLink we
243 decided to use a hybrid database in which the entire *B. subtilis* Uniprot protein database (4260
244 entries) was combined with the entire *Saccharomyces cerevisiae* Uniprot protein database (6043
245 entries). In this way we could get insight in the nature of false positives, i.e. nominations with one or
246 two yeast sequences, in order to find criteria to discriminate between true and false positives.

247 The result of a pLink search with the hybrid database is shown in Table 2. With the increase of the
248 search space while keeping the pLink 2 settings at 5% FDR, the numbers of inter-protein and intra-
249 protein peptide pairs overlapping with the Raeng cross-links dropped by about 10% (Table 2). Also
250 the number of homodimeric peptide pairs decreased. In addition, pLink 2 nominated several decoy
251 sequences. All decoy sequences contain peptide pair sequences from different proteins
252 (supplementary Table S3, column F, salmon-pink highlighted). Since no decoy sequences are present
253 with peptide pairs from the same yeast protein, the FDR for intra-protein target peptide pairs must
254 be very low. Previously we also noticed that only about 0.3% of all decoy hits consisted of intra-
255 protein target and reversed sequences in a complex sample of cross-linked human proteins [11]. This
256 justifies the inclusion of all intra-protein cross-links, along with the common set of inter-protein
257 cross-links put forward by Rang and pLink 2, in the dataset to calculate the mass and charge
258 distribution patterns in relation with the elution time in SCX chromatography (Table 1). It appeared
259 that some decoy sequences put forward by pLink 2 eluted in an unexpected time window. We use an
260 anomalous elution time during SCX chromatography as one of the criteria for filtering the dataset to
261 decrease the FDR for inter-protein cross-linked peptide pairs.

262 We also noticed that many decoy sequences showed relatively few y ions assigned in one or both
263 composite peptides. Furthermore ambiguity in the assignment of y or b ions occurred sometimes. A
264 relatively low matched intensity of MS/MS spectra was also not uncommon. Therefore we applied
265 thresholds for matched intensity and for the required number of unambiguously assigned y ions and
266 sometimes b ions as described in sections 2.3 and 2.4.

267 Another source of nominated decoy sequences are cases in which pLink 2 put forward 2 candidates
268 for the same precursor ion or for two different precursor ions with the same mass and eluting in the
269 LCMSMS run within a time window of 5 sec. In a few other instances alternative candidates were
270 also possible for a given precursor ion when certain post-translational modifications were taken into
271 account, namely formylation in two instances and carbamidomethylation at the N-terminus in three

272 instances. In supplementary Table S4 these double nominations are listed. In all cases only the best
273 scoring candidate was assigned, or, in case of equal scores, none of the candidates. In
274 supplementary Tables S2, S3, S5 and S6 these co-called ambiguous nominations that are not
275 assigned are cyan highlighted in column C. In future experiments, the otherwise rare
276 carbamidomethylation at the N-terminus can be circumvented by preventing the presence of
277 iodoacetamide during digestion by trypsin. Formylation is probably unavoidable [23] in the sample
278 preparation work flow where relatively high concentrations of ammonium formate are used to elute
279 peptides during SCX chromatography. To keep the formylation level as low as possible, the SCX
280 fractions were snap frozen in liquid nitrogen immediately after elution followed by lyophylisation

281 The aim of retention time window determination, application of thresholds for matched intensity
282 and for the numbers of unambiguously assigned y and b ions a, and exclusion of double nominations
283 for the same precursor ions is to lower substantially the FDR, while a high percentage of true
284 positive cross-linked pairs should survive the stringent criteria. Since intra-protein cross-links were
285 identified under conditions that no intra-protein decoy sequences were detected, these target cross-
286 links can be considered as true positives. Therefore intra-protein peptide pairs can be used to assess
287 the effect of filtering on the sensitivity of assignment of true positive inter-protein cross-linked
288 peptide pairs.

289 *3.5. Application of mass spectrometric and SCX chromatographic criteria to obtain a low FDR for*
290 *inter-peptide protein pairs*

291 The effect of application of the criteria as formulated in the Material and Methods sections 2.3 and
292 2.4 and discussed in section 3.4 is shown in Table 2 (column assigned target peptide pairs and
293 column assigned decoy sequences). For details see columns H-J in supplementary Tables S2 and S3).
294 Only one decoy sequence remains that fulfils the criteria, corresponding to a FDR of about 2% for
295 non-redundant inter-protein peptide pairs (Supplementary Table S3). With one exception the
296 nominated inter-protein peptide pairs of which at least one of the α or β peptide did not belong to

297 the 673 independently identified proteins in the sample were all rejected by the filtering
298 (supplementary Table S2). This shows that the composite filter that we developed is very effective to
299 prevent suspicious candidates from assignment.

300 Application of the composite filter to the intra-protein cross-linked peptide pairs identified by pLink
301 2 from the entire *B. subtilis* database shows that 77% fulfilled the criteria for assignment of inter-
302 protein peptide pairs (supplementary Table S2, columns G, H and I). This shows that also true
303 positive cross-linked peptide pairs revealing protein-protein interactions can be detected at a very
304 low FDR and high sensitivity.

305 The small drop in the number of identified peptides when the size of the database increase
306 prompted to interrogate a much smaller database with pLink 2, in the expectation to identify some
307 more cross-linked peptide pairs. In this case the protein database to be searched was composed of
308 the 673 independently identified proteins in the sample. For decoy sequences 1085 human proteins
309 were added to the *B. subtilis* set of proteins. Nominated and identified cross-links and decoy
310 sequences are listed in Supplementary Tables S5 and S6. The results are summarized in Table 2,
311 showing a small increase in the number of assigned inter-protein and intra-protein cross-linked
312 peptide pairs as compared with the results obtained by interrogation of the entire *B. subtilis*
313 sequence database.

314 *3.6. Resemblances and differences of results and approaches between pLink 2 and Raeng/Mascot*

315 The combined searches showed that the overlap between the identifications by Raeng/Mascot and
316 pLink 2 is about 80%. In supplementary figure 1 pLabel-generated mass spectra are depicted with
317 input of the mgf files of the inter-protein cross-linked peptide pairs that had escaped detection by
318 pLink 2. All spectra fulfilled the criteria for assignment. On the other hand pLink identified several
319 cross-linked peptide pairs that had been overlooked by Raeng/Mascot (see for 4 examples the mass
320 spectra in supplementary figure 2). The amount of cross-links put forward by pLink 2 and Raeng may

321 depend on how exhaustive pLink 2 searches for the right peptide masses of α and β, sometimes
322 against a background of secondary fragment pairs differing 125.048 Da, and how exhaustive it
323 searches for their identities, while in the Raeng/Mascot approach the suitability of Mascot to find
324 candidate sequences for α and β under the given conditions may by limiting.

325 *3.7. New PPIs detected by pLink 2*

326 The new cross-links identified by pLink 2 revealed 6 PPIs not identified in the Raeng/Mascot
327 approach. One cross-linked peptide pair points to the interaction by which enzyme I (PT1_BACSU)
328 from the sugar PTS system transfers a phosphoryl group from phosphoenolpyruvate (PEP) to the
329 phosphoryl carrier protein HPr (PTHP_BACSU) [24]. Another cross-link reveals an interaction
330 between the RNA chaperone CspB (CSBP_BACSU) and ribosomes, in particular with the 50S
331 ribosomal protein L7/L12 (RL7_BACSU). Previously we found already a cross-link between CspB and
332 the 30S ribosomal protein S2 [9]. This shows that CspB acts in close proximity of ribosomes,
333 corroborating other observations, although a direct interaction with ribosomes could not be
334 demonstrated before [25]. Four cross-links point to novel PPIs (Table 4). In supplementary Fig. 2 the
335 corresponding mass spectra are depicted. One PPI not reported up to now as far as we know is
336 revealed by a cross-linked peptide pair from the transition state regulatory protein AbrB
337 (ABRB_BACSU) and translation elongation factor Tu (EFTU_BACSU). AbrB is known for its DNA
338 binding activity of a large number of sites [26,27]. It can be phosphorylated by several protein
339 kinases and it interacts with the anti-repressor AbbA [28–30]. The physiological significance of the
340 interaction of AbrB with elongation factor Tu is not clear. AMPA_BACSU is a cytosolic
341 aminopeptidase with broad specificity [31]. The interaction with the 50S ribosomal protein L17
342 (RL17_BACSU), which is located in close vicinity of the site where the nascent polypeptide emerges
343 form the ribosome at the end of the tunnel [32] raises the question whether AmpA plays a role in
344 either the removal of aminoterminal (formyl)-methionine during protein synthesis along with the
345 canonical methionine aminopeptidases MAP and YflG [33,34] or in another post-translational

346 modification. The only inter-protein cross-linked peptide pair of which one of the proteins (YopJ) is
347 not a member of the 673 independently identified proteins, revealed an interaction with the α-
348 subunit of RNA polymerase. The function of YopJ, a SPbeta prophage-derived protein, is not known.
349 An intriguing interaction is found between the essential protein YlaN (YLAN_BACSU) [35] and the
350 ferric uptake repressor Fur (FUR_BACSU) [36]. The function of YlaN has long been an enigma, but
351 recent data point to a role in FeS cluster biogenesis [37]. The interaction of Ylan with Fur as shown
352 here may provide a clue to understand how the effect of Ylan on FeS cluster biogenesis is brought
353 about.

354 *3.8. Contributions of the different assignment criteria to obtain a low FDR for inter-protein peptide*
355 *pairs.*

356 In Table 5the contributions are listed of the different criteria used to discriminate between true and
357 false positives. Details are depicted in Supplementary Tables S7 and S8. In the majority of cases the
358 acceptance of assignment of a decoy sequence can only be prevented based on a single criterion,
359 most decoy candidates passing the thresholds for the other criteria. The most discriminating
360 criterion is the number of unambiguously assigned y ions. Unambiguousness in this respect is crucial,
361 since it can significantly lower the FDR as shown in Table 5. The dependence of the elution time
362 window during SCX chromatography on mass and charge of cross-linked peptide pairs is also a
363 powerful criterion to identify a significant fraction of false positive inter-protein peptide pairs. SCX
364 fractionation is often used for cleavable and non-cleavable cross-linkers using 1D [34–36]
365 or 2D approaches [41]. The usually large population of intra-protein cross-linked peptide pairs can
366 be used to prepare a set of references for SCX elution time windows. Here and previously [11,12] we
367 have shown that the chance that intra-protein cross-link pairs are found by accident by searching an
368 entire species specific sequence database is so small that false positives will be rare in this category
369 of cross-links .

370

371 *3.9. A search at 0.1% FDR also requires additional filtering to obtain a low FDR for inter-protein*
372 *peptide pairs*

373 Finally the dataset was searched at 0.1% FDR by pLink 2 against the entire *B. subtilis* database. The
374 data are depicted in Table 2. Slightly less candidates were nominated for both inter-protein peptide
375 pairs, inter-protein peptide pairs and homo-dimer peptide pairs than in the search at 5% FDR.
376 However, compared with the search at 5% FDR, exactly the same number and same identity of inter-
377 protein peptide pairs were assigned after application of the composite filter. Only a few less
378 candidates with aberrant SCX elution times or with one or both peptides not belonging to the 673
379 independently proteins were rejected by applying the composite filter than in the 5% FDR search.
380 The relatively large number of rejected spurious candidates in a search with an overall FDR as low as
381 0.1%, underscores the usefulness of the composite filter to obtain a low FDR for inter-protein
382 peptide pairs during interrogation of a large sequence database.

383

384 **4. Conclusions**

385 Here we show that pLink 2 efficiently nominated cross-linked peptide pairs from complex protein
386 extracts after in vivo treatment of exponentially growing cells with BAMG. pLink 2 is also extremely
387 fast as compared with use of our in house developed program Raeng for nomination of BAMG-cross-
388 linked peptides. However, at an overall FDR of 5% or 0.1%, additional filtering is required to obtain a
389 low FDR for inter-protein cross-linked peptide pair identifications. This is due to the notion that false
390 positives are practically all confined to inter-protein peptide pairs when a search space as large as an
391 entire species specific database is interrogated at a given FDR, if equal criteria for assignment of
392 intra-protein and inter-protein peptides are used [11]. This can be circumvented by applying more
393 stringent criteria for inter-protein cross-linked peptide pairs than for intra-protein cross-links. Here
394 we show that matched intensity, the number of y ions to be assigned for both α and β and the

unambiguousness of y ions, and to a lesser extent, b ions are useful criteria to diminish the FDR for inter-protein cross-linked peptide pairs. For future use of pLink 2 as a search engine for BAMG-cross-linked peptides pairs it would be useful if these criteria could be implemented in the pLink 2 code for cross-link approval. Also the number of assigned b ions may be included in the filter criteria, but with a correction factor for the relatively low average abundance as compared with the abundance of y ions. The isolation method used for BAMG-cross-linked peptides, diagonal SCX chromatography, offers an additional criterion for filtering based on their mass and charge at the pH of the chromatographic mobile phase in relation to the elution time.

About 20% high scoring candidates identified previously with Raeng had escaped detection by pLink 2, and vice versa. While pLink 2 is fast and efficient, identifying approximately the same number of cross-linked-peptide pairs as compared with our previous approach, it would be worthwhile to understand the reasons for the slightly difference in output between these two approaches for the benefit of future research with BAMG and other cleavable cross-linkers.

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567

568 Table 1. Charge at pH 3 and mass distributions of cross-linked peptide pairs in SCX fractions

SCX fraction	XL peptide pairs (n)	Mass range (Da) at charge +3	XL peptide pairs (n)	Mass range (Da) at charge +4	XL peptide pairs (n)	Mass range (Da) at charge +5	XL peptide pairs (n)	Mass range (Da) at charge 6+	XL peptide pairs (n)
7	30	1793-1868	2	2274-4505	28				
8	62	1869-2109	2	2274-4387	59	4377	1		
9	212			1756-3890	201	3273-4672	11		
10	72			1641-3033	69	2554-3394	3		
11	83			1734-2858	45	2861-5181	36	4055-6092	2
12	57			1513-2568	17	2540-4462	39	2661	1
13	52			1562-2090	5	2262-3828	46	3884	1
14	39			1435-1779	4	1967-3351	24	3419-4919	11
15	2					1862-2595	2	3405	1
16	17					1862-3510	13	3313-3870	4

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572 Table 2. Overview of non-redundant spectral counts of nominated and assigned cross-linked peptide pairs

database	cross-linked peptide pair type	non-redundant spectral counts					
		Raeng			pLink 2		
		assigned target peptide pairs	nomin-ated target peptide pairs	over-lap with Raeng	assigned target peptide pairs	nomin-ated decoy sequen-ces	assigned decoy sequen-ces
target; *B. subtilis,* 4620 proteins; 5% FDR	inter-protein	55	89	40	53		
	intra-protein	335	449	284	449		
	homodimer	24	13	11	13		
target/decoy; *B. subtilis* 4620 proteins/*S. cerevisiae* 6049 proteins; 5% FDR	inter-protein		59	36	46	29	1
	intra-protein		401	278	401	0	0
	homodimer		6	4	6	0	0
target; *B. subtilis,* 673 proteins; 5% FDR	inter-protein		88	44	58		
	intra-protein		463	n.d.	463		
	homodimer		7	n.d.	7		
target/decoy; *B. subtilis* 673 proteins/*H. sapiens* 1085 proteins; 5% FDR	inter-protein		50	40	45	30	0
	intra-protein		450	n.d.	450	0	0
	homodimer		7	n.d.	7	0	0
target; *B. subtilis,* 4620 proteins, 0.1% FDR	inter-protein		85	40	53		
	intra-protein		444	n.d.	444		
	homodimer		10	n.d.	10		

573 n.d., not determined

574

575 Table 3. Overview of spectral counts identified by pLink 2 at <5% FDR

SCX fraction	Counts					
	Cross-linked spectra		Loop-linked spectra	Mono-link spectra	Regular peptide spectra	Total spectra
	Intra-protein	Inter-protein				
7	94	3	183	566	1185	2031
8	124	16	143	478	779	1540
9.2	12	9	2	30	138	191
9.3	268	39	50	251	161	769
9.4	254	19	103	305	185	867
9.5	32	2	74	136	105	349
10	174	23	53	150	404	803
11	229	10	33	172	441	885
12	173	17	18	130	268	606
13	146	99	64	133	392	834
14	66	7	37	47	320	477
15	6	1	0	4	172	183
16	26	7	10	22	257	322
total	1604	252	770	2424	4807	9857
percent	16.3	2.6	7.8	24.6	48.8	

576

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579
580 Table 4. Cross-linked peptide pairs identified by pLink 2 revealing new protein-protein interactions

SCX fract-ion	peptide sequences; numbering of linked K between brackets	mass (Da)	ID of linked proteins; numbering of linked K between brackets	mass spect-rum	M.I. (%)	Y ions α	Y ions β
9.3	KEWEDVVGLVGK(1)-SVVEKMITLGK(5)	2704	sp|O32106|AMPA_BACSU (250)-sp|P20277|RL17_BACSU (46)	Suppl. Fig. 2a	77	7	6
9.4	KLLDYAEAGDNIGALLR(1)-KVDELGR(1)	2773	sp|P33166|EFTU_BACSU (266)-sp|P08874|ABRB_BACSU (11)	Suppl. Fig. 2b	84	11	5
9.4	FAKDIAEEVYYSLK(3)-KSLEEVK(1)	2632	sp|O31928|YOPJ_BACSU (127)-sp|P20429|RPOA_BACSU (294)	Suppl. Fig. 2c	74	6	5
9.3	VVDKINFGDGVSR(4)-VDAEKILK(5)	2445	sp|P54574|FUR_BACSU (74)-sp|O07638|YLAN_BACSU (23)	Suppl. Fig. 2d	72	10	3

581 M.I., matched intensity
582
583

584
585 Table 5. Contribution of different criteria used to obtain a low FDR for inter-protein cross-linked
586 peptide pairs

	Decisive contribution to rejection of hits			
	decoy hits		target hits	
Criterion	n	percent	n	percent
Matched intensity	1	1.7	5	6.9
Required number of y ions	16	27.1	18	25.0
Required number of b ions	2	3.4	3	4.2
Unambiguous assignment to y or b ions	6	10.2	8	11.1
Expected SCX elution time window	5	8.5	7	9.7
Combination of two or more of the above criteria	22	37.3	26	36.1
Better scoring candidate with the same mass and elution time in LCMSMS	3	5.1	3	4.2
Correction for formylation or carbamidomethylation	3	5.1	2	2.8
Assigned decoy sequence	1	1.7		
Total	59		72	

587

588 **Legends to Figures**

589

590 Figure 1. Gas phase cleavage reactions of BAMG-cross-linked peptides in which the azido group has
591 been reduced to an amine group. Upper left corner, structure of BAMG. Middle part, collision
592 induced dissociation (CID) of a cross-linked peptide pair leads to cleavages of the two cross-link
593 amide bonds along with cleavages of peptide bonds resulting in y an b ions. Cleavage of an amide
594 bond probably occurs by nucleophilic attack of the amine in the spacer of the cross-link to a
595 protonated carbonyl group of the amide bond. This leads to formation of an unmodified peptide or
596 short version of the cleavage product (αS or βS), the other peptide being modified by the remnant of
597 the cross-linker in the form of a γ lactam adding 125.048 Da to the mass of the peptide. This is the
598 longer version of the cleavage product (αL or βL). Amino acids are depicted as colored candies. The
599 indicated gas phase charge states of the cross-linked peptide and the cleavage products are
600 arbitrarily. The lower part is a cartoon of a fragment mass spectrum with two pairs of cleavage
601 products with the characteristic 125.048 Da mass difference (purple sticks) and some peaks of b
602 (green) and y (red) ions.

603

604 Figure 2. Size exclusion chromatogram on a Superose 6 10/300 GL column of a soluble extract from
605 Bacillus subtilis cross-linked in vivo with BAMG. MW markers, 443 kDa, apoferritine (horse spleen);
606 240 kDa catalase (bovine liver); 150 kDa, alcohol dehydrogenase (yeast); 66 kDa, bovine serum
607 albymine, 30 kDa carbonic anhydrase (bovine erythrocytes). Fractions are indicated by green
608 vertical lines. Duplicate chromatograms are shown by red and blue lines. Material in fraction 4-7 was
609 used in this study.

610

611 Figure 3. Workflow from in vivo cross-linking with BAMG to LCMSMS. Left part, 5 min after addition
612 of BAMG to an exponentially growing Bacillus subtilis culture the cross-linker is quenched and cells
613 are harvested and sonicated. (1), the cross-linked protein extract is subjected to SEC and then

614 digested to obtain a peptide mixture with cross-linked α-β peptide pairs. Right part, the peptide
615 mixture is fractionated by strong cation exchange chromatography (first dimension SCX), using a
616 mobile phase of pH 3 and a salt gradient (red lines) of ammonium formate to elute bound peptides.
617 Grey, regular peptides; cyan, cross-linked peptides. (2), reduction by TCEP of the azido group in the
618 spacer of the cross-linker to an amine group in selected SCX fractions. At the pH of the mobile phase
619 of SCX chromatography the amino group is protonated adding an extra positive charge to cross-
620 linked peptides. The TCEP-treated primary fractions are then separately subjected to the secondary
621 runs of SCX. Here target peptides are sequestered from the bulk of unmodified peptides that elute at
622 the same time as in the primary run, while the extra charge state of the cross-link peptides leads to
623 elution at a later time. Depicted peptide charge states after (1) and (2) are calculated for pH 3,
624 assuming full protonation of the two amino-termini plus 2 basic amino-acid side chains, carboxylic
625 acid side chains being uncharged under these conditions.

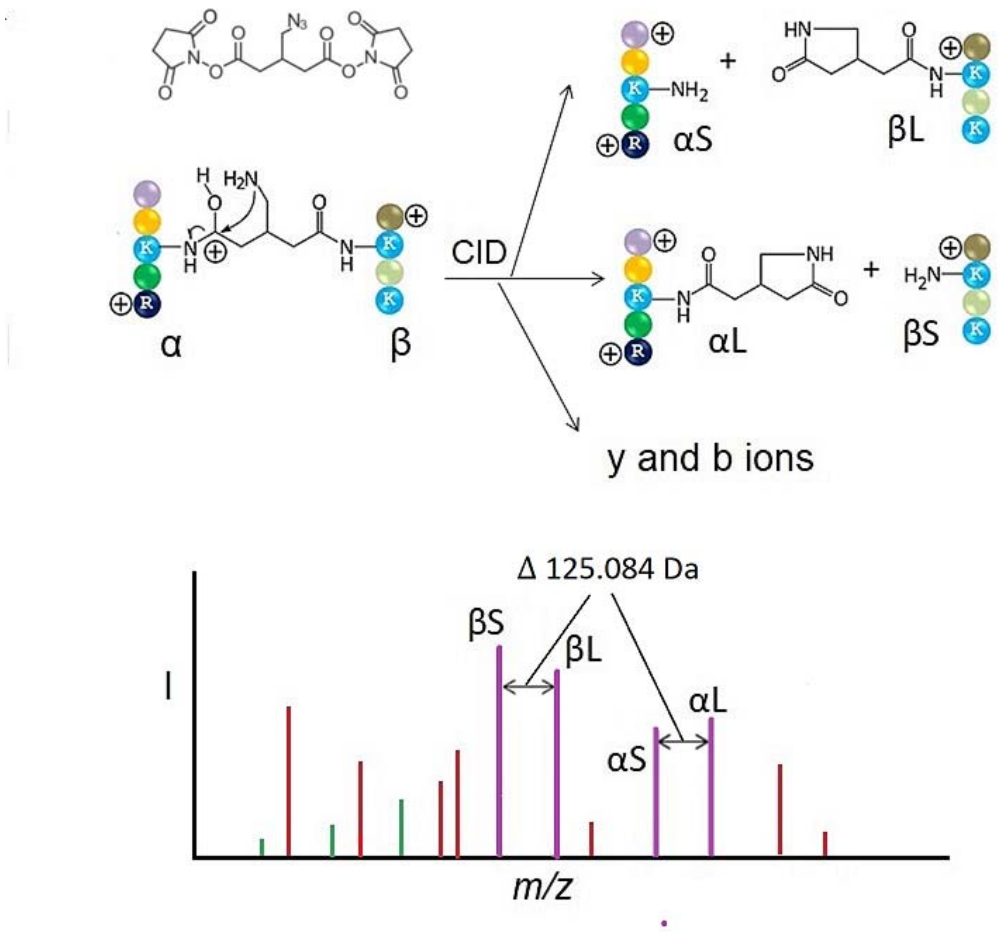
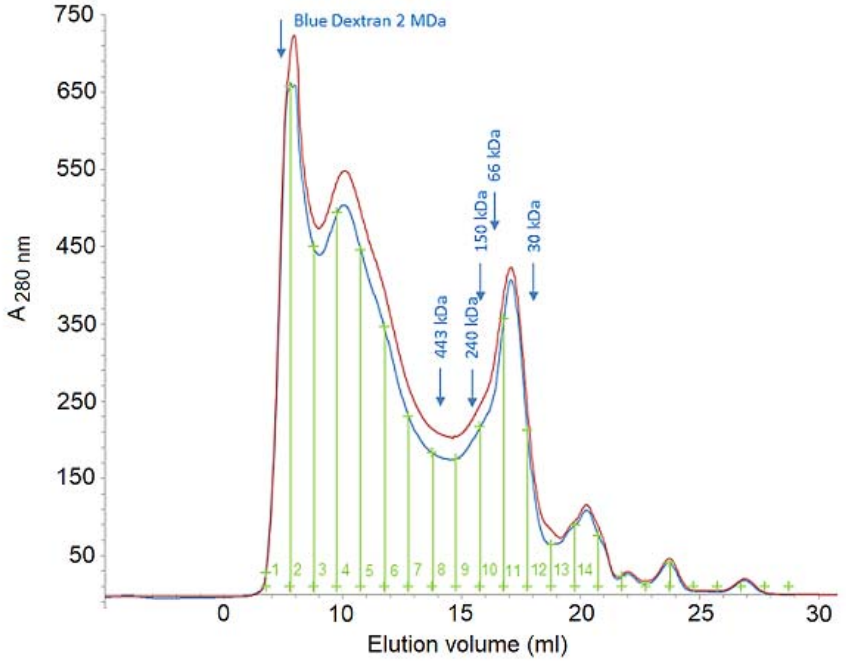


Fig 1.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443 kDa
240 kDa
150 kDa
66 kDa
30 kDa
A 280 nm
750
650
550
450
350
250
150
50
0
10
15
20
25
30
Elution volume (ml)
1
2
3
4
5
6
7
8
9
10
11
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13
14

628

629 Fig. 2

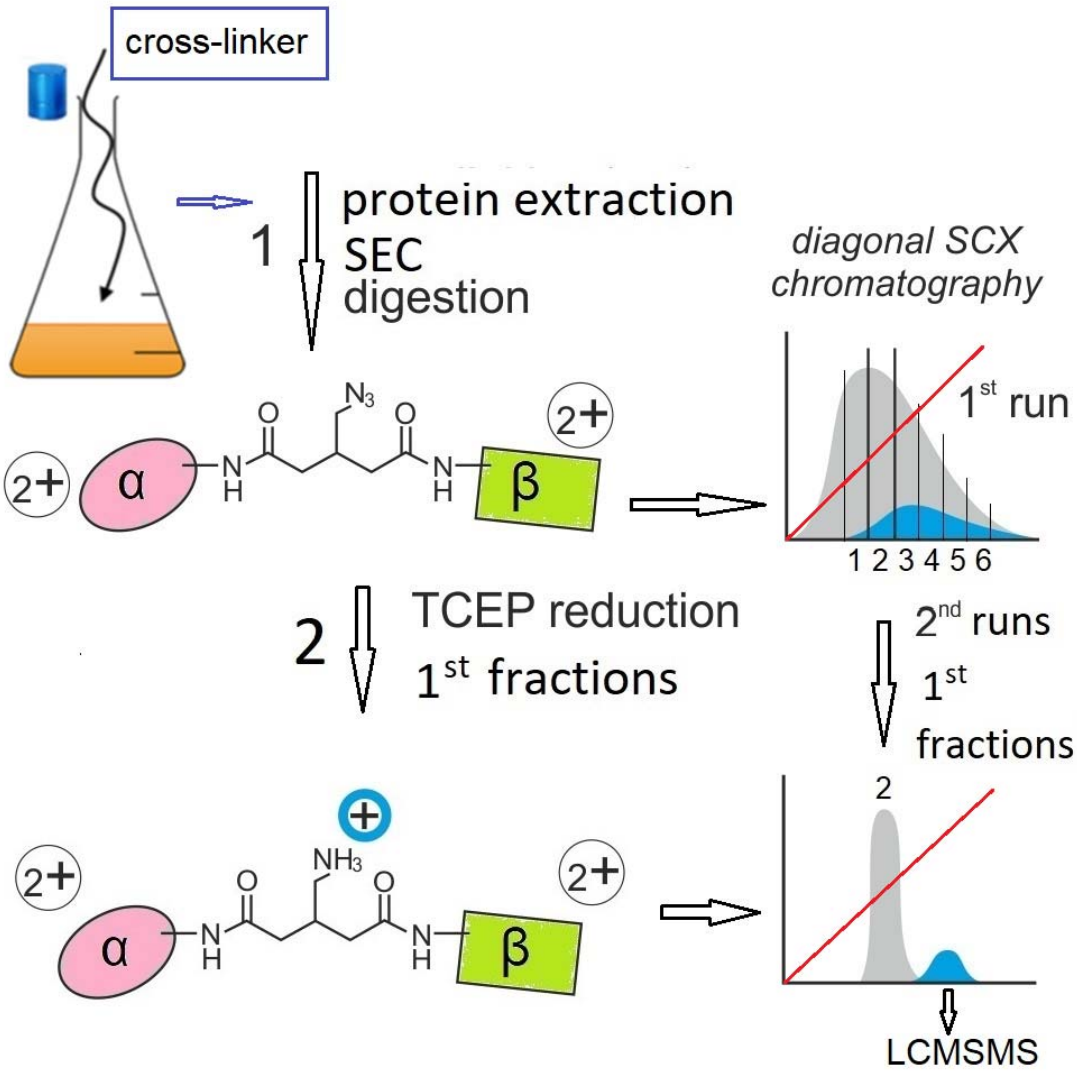

cross-linker
1
protein extraction
SEC
digestion
diagonal SCX
chromatography
1st run
1 2 3 4 5 6
2+
α
β
2
TCEP reduction
1st fractions
2nd runs
1st
fractions
2
LCMSMS

Fig 3.

633 **Supplementary material**

634 Table S1

635 Legends to Supplementary figures

636 Supplementary Figure 1

637 Supplementary Figure 2

638

639

640 Supplementary Table S1. Mgf files used in this study

mgf file used in this study	combined from original mgf files	primary SCX fraction	Size (MB)	mass accuracy (ppm) for both precursor and fragments ions
7.mgf	7.1.1, 7.2.1 and 7.3.1	7	31.9	25
8.mgf	8.1.1, 8.2.1. and 8.3.1	8	21.7	25
9.2. mgf		9	6.0	75
9.3.mgf		9	19.2	50
9.4.mgf		9	25.5	75
9.5.mgf		9	16.1	30
10.mgf	10.1.1, 10.2.1 and 10.3.1	0	17.5	25
11.mgf	11.1.1, 11.2.1 and 11.3.1	1	18.3	25
12.mgf	12.1.1, 12.2.1 and 12.3.1	2	10.4	25
13.mgf	13.1.1, 13.2.1 and 13.3.1	3	12.7	25
14.mgf	14.1.1, 14.2.1 and 14.3.1	4	8.2	25
15.mgf	15.1.1, 15.2.1 and 15.3.1	5	0.8	25
16.mgf	16.1.1, 16.2.1 and 16.3.1	6	4.4	25

641

642

643

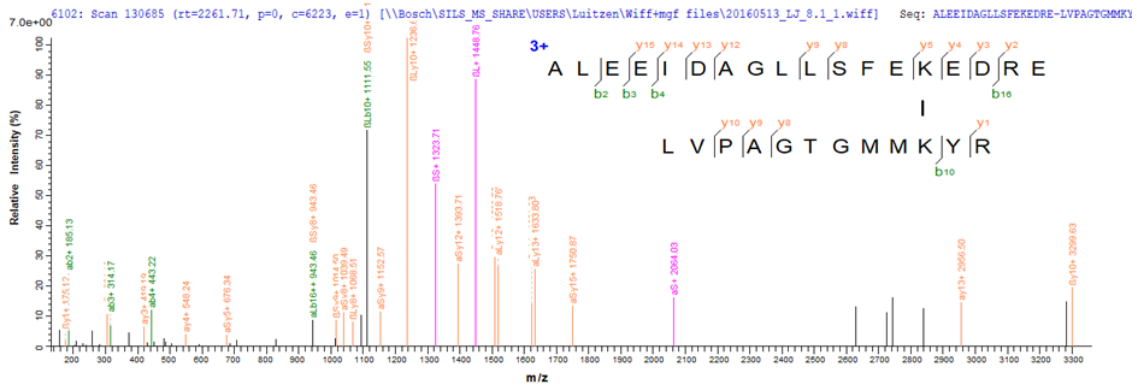
644 **Legends to supplementary figures**

645 Supplementary figure 1. Mass spectra with high scores of inter-protein cross-linked pairs identified
646 by Raeng/Mascot, but not pLink 2 showing interactions between a, RPOZ_BACSU and RPOC_BACSU;
647 b, SIGA_BACSU and RPOB_BACSU; c, RPOB_BACSU and SIGA_BACSU; d, GREA_BACSU and
648 RPOC_BACSU; e, RL11_BACSU and RL7_BACSU; f, RL11_BACSU and RL7_BACSU; g, RL31_BACSU and
649 RS13_BACSU; h, GLTA_BACSU and GLTB_BACSU; i, ODPA_BACSU and ODPB_BACSU; j, RS10_BACSU
650 and RS18_BACSU

651 Supplementary figure 2. Mass spectra of cross-linked peptide pairs identified by pLink 2 revealing
652 new protein-protein interactions a, AMPA_BACSU-RL7_BACSU; b, EFTU_BACSU-ABRB_BACSU; c,
653 YOPJ_BACSU-RPOA_BACSU; d, FUR_BACSU-YLAN_BACSU
654

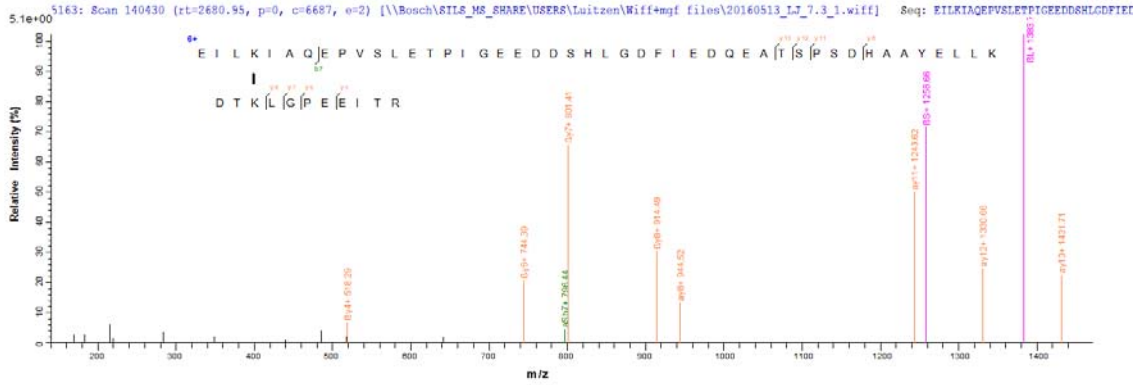
Supplementary Figure 1. Mass spectra of intra-protein cross-linked pairs identified by Raeng?Mascot, but not by pLink 2.

a



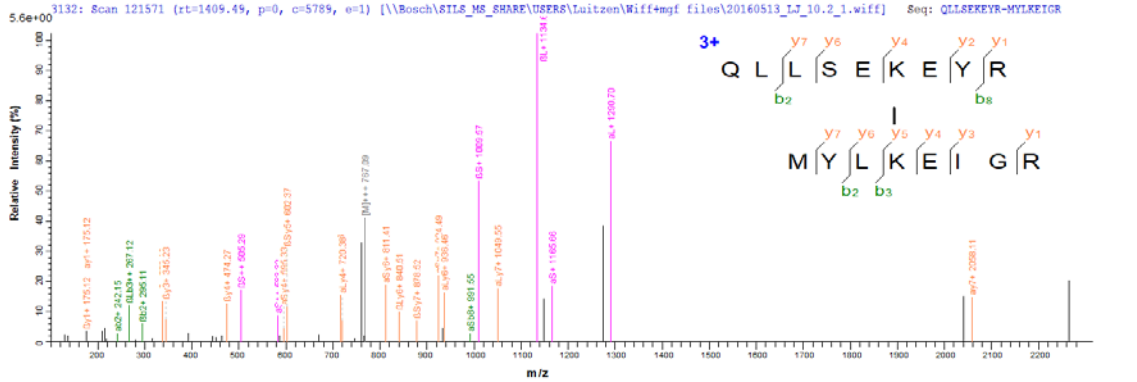
Cross-link between RPOZ_BACSU and RPOC_BACSU; matched intensity 83.1%

b



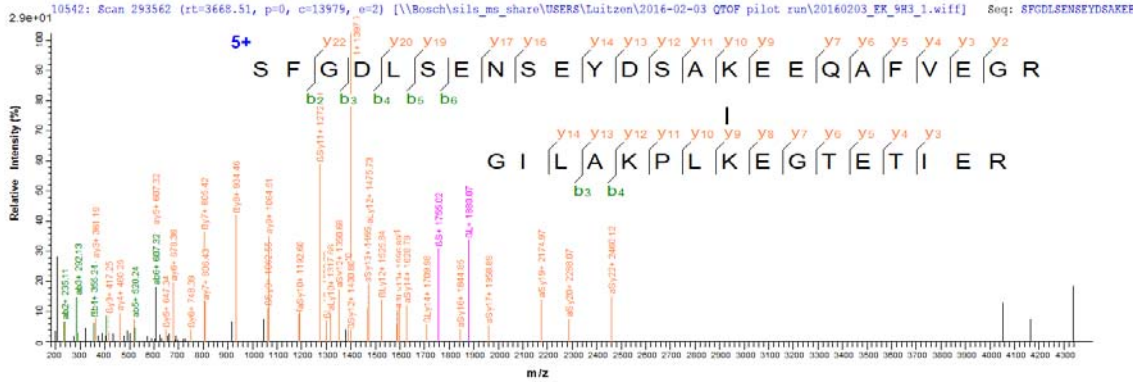
Cross-link between SIGA_BACSU and RPOB_BACSU; matched intensity 93.8%

c



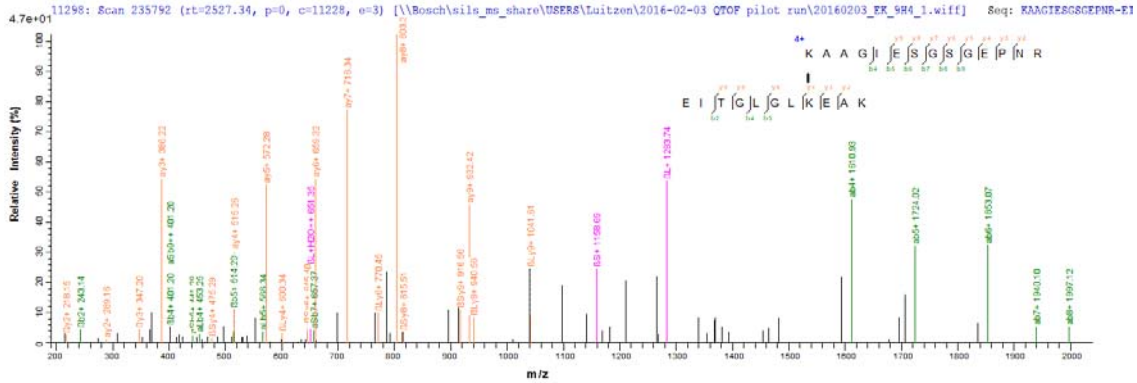
Cross-link between RPOC_BACSU and SIGA_BACSU; matched intensity 58.1%

d

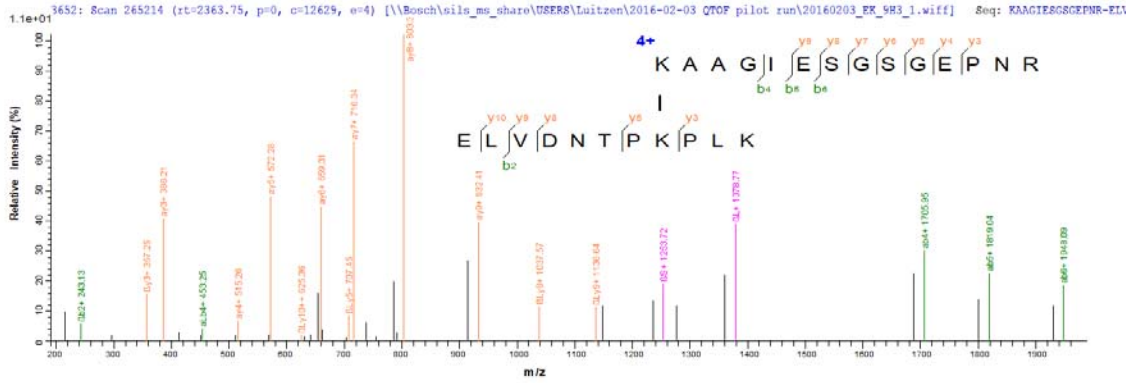


Cross-link between GREA_BACSU and RPOC_BACSU; matched intensity 82.3%

e

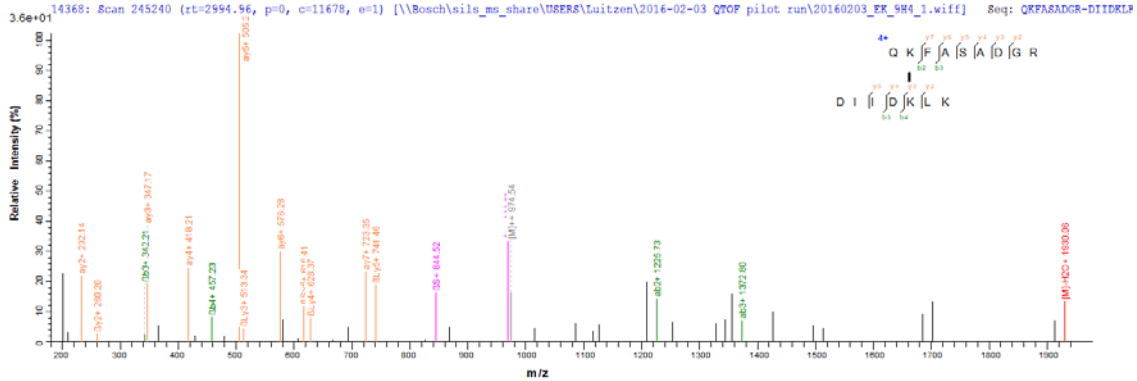


Cross-link between RL11_BACSU and RL7_BACSU; matched intensity 62.0%



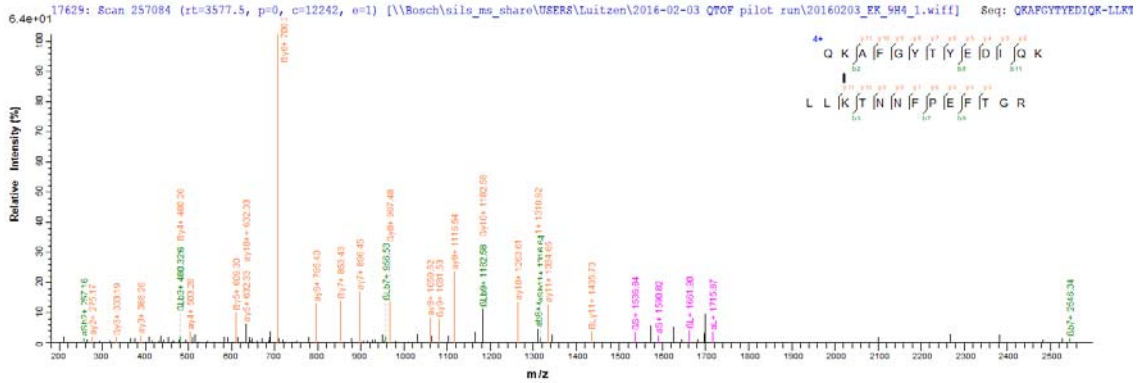
Cross-link between RL11_BACSU and RL7_BACSU; matched intensity 70.1%

g



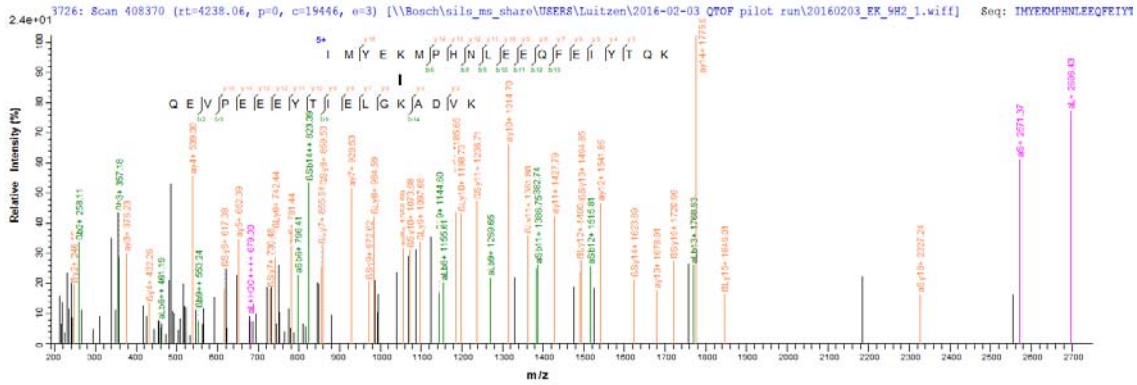
Cross-link between RL31_BACSU and RS13_BACSU; matched intensity 65.2%

h



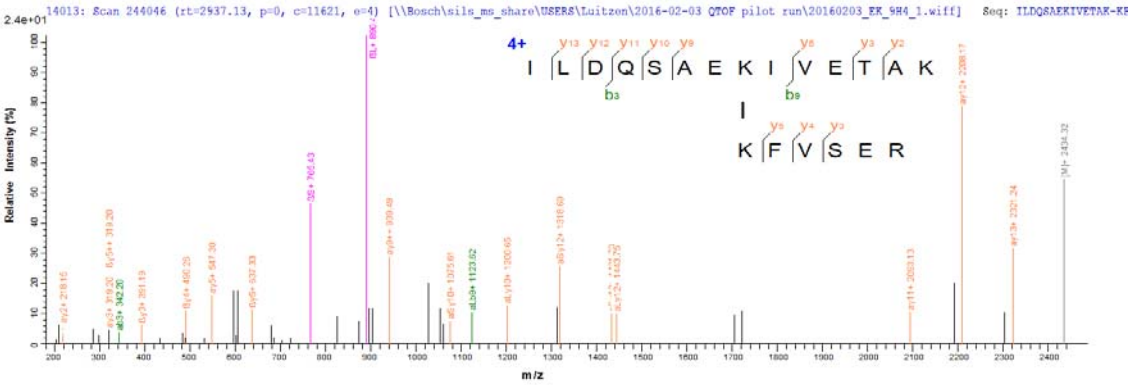
Cross-link between GLTA_BACSU and GLTB_BACSU; matched intensity 74.5%

i



Cross-link between ODPA_BACSU and ODPB_BACSU; matched intensity 58.1%

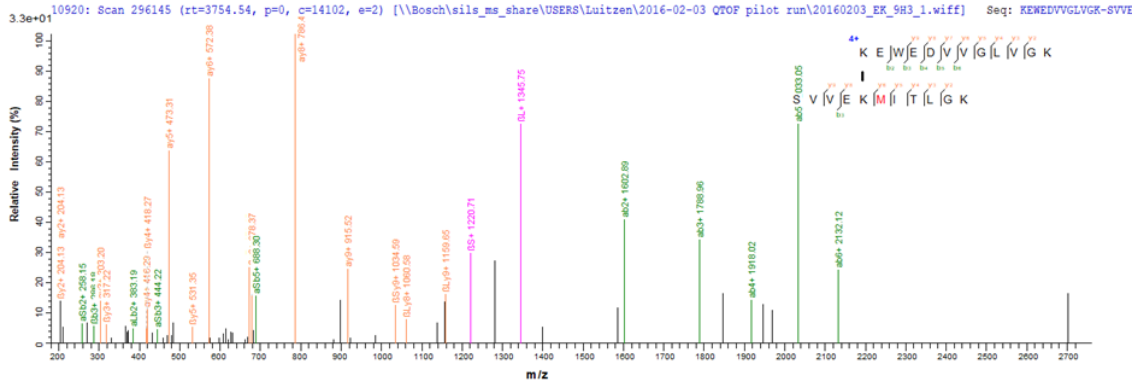
j



Cross-link between RS10_BACSU and RS18_BACSU; matched intensity 67.2%

Supplementary Figure 3. Mass spectra of cross-linked peptide pairs revealing new protein-protein interaction a, AMPA_BACSU-RL7_BACSU; b, EFTU_BACSU-ABRB_BACSU; c, YOPJ_BACSU-RPOA_BACSU; d, FUR_BACSU-YLAN_BACSU; see also Table 4.

a



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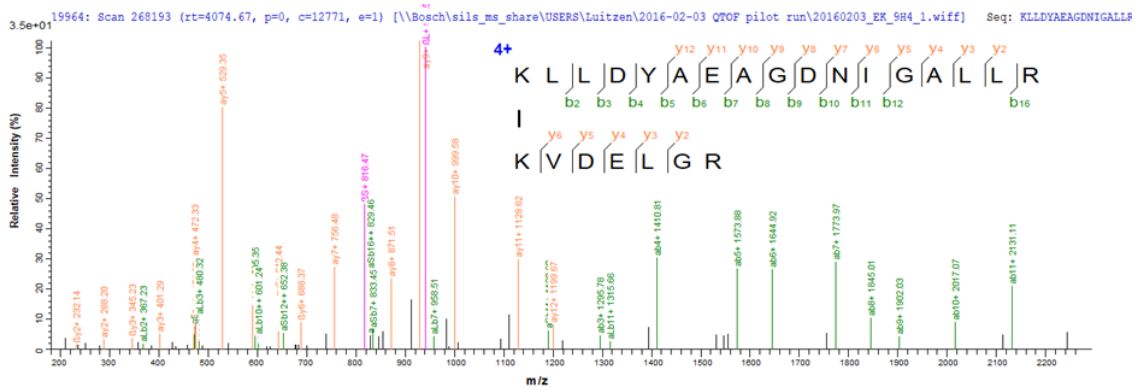

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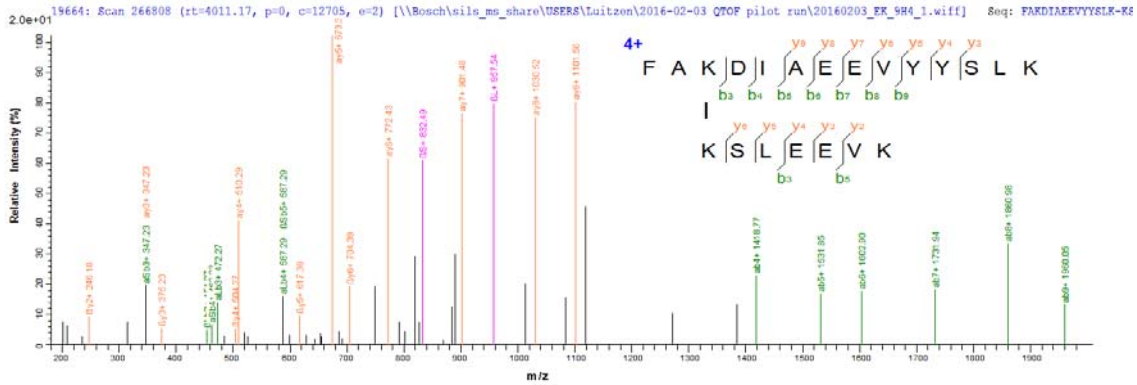

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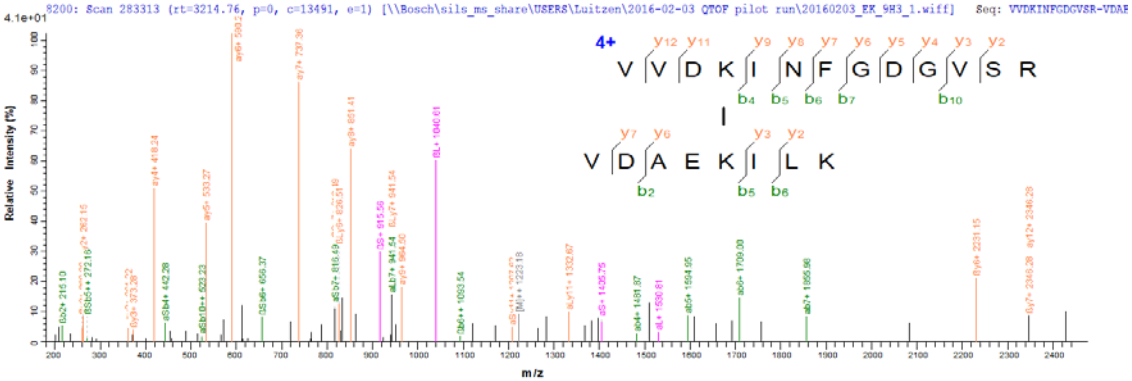
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4.1e+01
Relative Intensity (%)
m/z
4+
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y12 y11 y9 y8 y7 y6 y5 y4 y3 y2
b4 b5 b6 b7 b10
I
V D A E K I L K
y7 y6 y3 y2
b2 b5 b6

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