

# CLIP: A cross-linker for enrichment and confident identification of protein cross-linking sites by mass spectrometry

Saiful M. Chowdhury<sup>1</sup>, Xiuxia Du<sup>2</sup>, Nikola Tolić<sup>1</sup>, Ashoka P. Polpitiya<sup>1</sup>, Ronald J. Moore<sup>1</sup>, John R. Cort<sup>1,3</sup>, M. Uljana Mayer<sup>1</sup>, Richard D. Smith<sup>1</sup>, Joshua N. Adkins<sup>1</sup>  
<sup>1</sup>Pacific Northwest National Laboratory, Richland, WA; <sup>2</sup>University of North Carolina, Charlotte, NC; <sup>3</sup>Washington State University, Tri-Cities, Richland, WA



Pacific Northwest  
NATIONAL LABORATORY

## Overview

A compact chemical cross-linker, CLIP (click enabled cross-linker for interacting proteins) with enrichment functionality was designed and synthesized. Sequential CID and ETD-MS/MS is demonstrated to aid in the unambiguous identification of cross-linked peptides.

## Introduction

- Chemical cross-linking combined with mass spectrometry can be a powerful approach for the identification of protein-protein interactions and for providing constraints on protein structures.
- However, enrichment of cross-linked peptides is crucial to reduce sample complexity before mass spectrometric analysis. In addition, compact cross-linkers are often preferred to provide short spacer lengths, surface accessibility to the protein complexes, and must have reasonable solubility.
- We present a novel compact cross-linker that contains two distinct features: 1) an alkyne tag and 2) a small molecule detection tag ( $\text{NO}_2^-$ ). The alkyne tag enables enrichment of the cross-linked peptide after proteolytic cleavage after coupling of an affinity tag using alkyne-azido click chemistry. Neutral loss of the small  $\text{NO}_2^-$  moiety provides a secondary means of detecting cross-linked peptides in MS/MS analyses, providing additional confidence in peptide identifications.

## Methods

- Labeling efficiency of CLIP was demonstrated with Ubiquitin. Enrichment capability was demonstrated in high complexity *E. coli* cell lysate spiked with different ratios of cross-linked Ubiquitin.
- LC-MS/MS analysis was performed using LTQ/LTQ-Orbitrap mass spectrometer. MS/MS spectra were analyzed using Xlink-identifier developed in house. Inter cross-linked peptides (two peptides connected with cross-linker) were manually validated using another software Xlink-explorer.

## Results

### Cross-linker structure and enrichment strategy

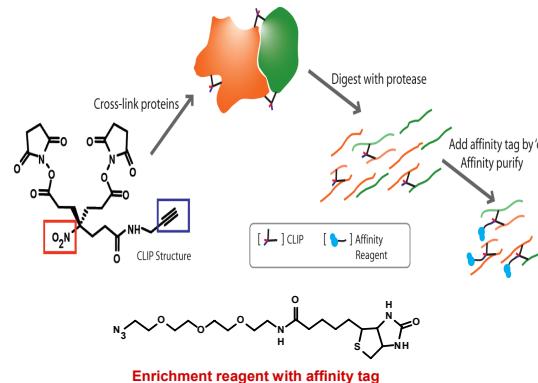


Figure 1: Clip structure and enrichment strategy of cross-linked peptides after addition of an affinity tag through alkyne-azido click chemistry (alkyne tag for enrichment, blue rectangle, and  $\text{NO}_2$  tag for neutral loss validation, red rectangle).

### CLIP cross-linking and CID-MS/MS before enrichment

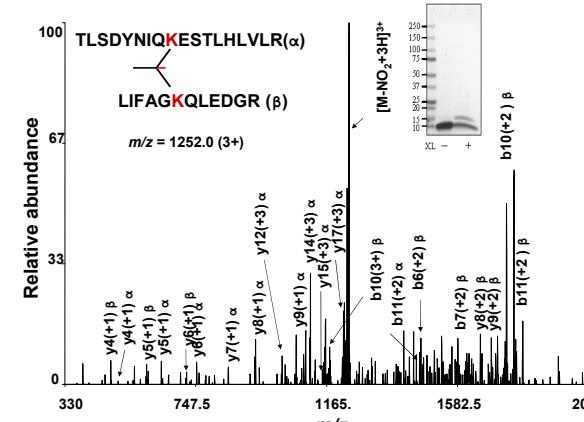


Figure 2: Example of an inter cross-linked peptide identified from cross-linking reaction of Ubiquitin before enrichment. Ubiquitin was cross-linked with CLIP and digested prior to LC-MS/MS.

### CLIP cross-linking and CID MS/MS after coupling with enrichment reagent

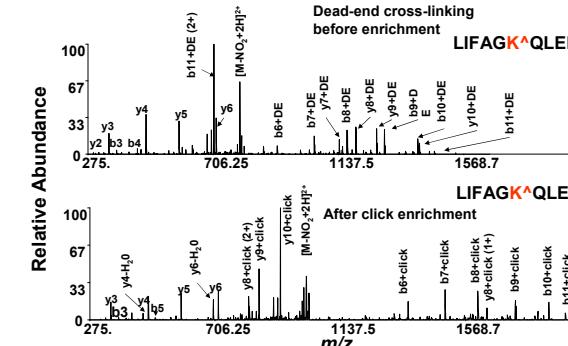


Figure 3: Cross-linker stability with and without the enrichment reagent during MS/MS of a dead-end modified peptide from Ubiquitin.

### Enrichment of cross-linked species from complex background

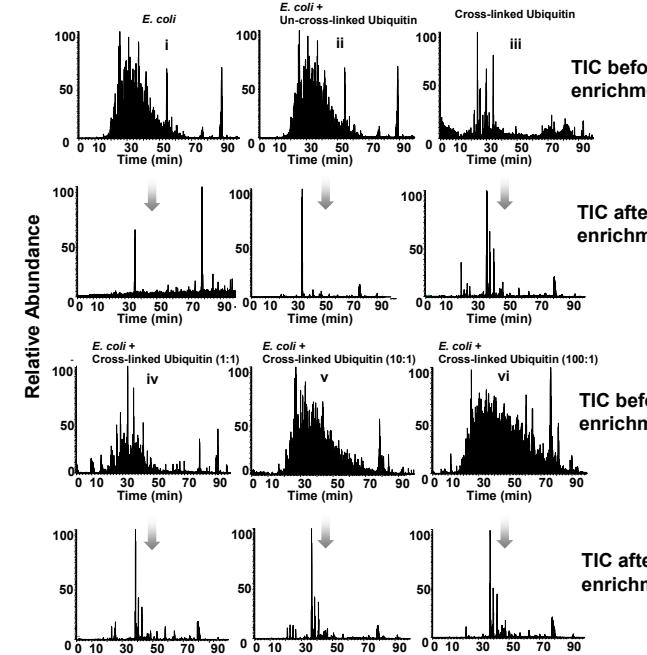


Figure 4: Enrichment of cross-linked peptides from high complexity *E. coli* cell lysate spiked with different ratios of cross-linked Ubiquitin.

### Application of sequential CID-MS/MS and ETD-MS/MS after enrichment of cross-linked peptides

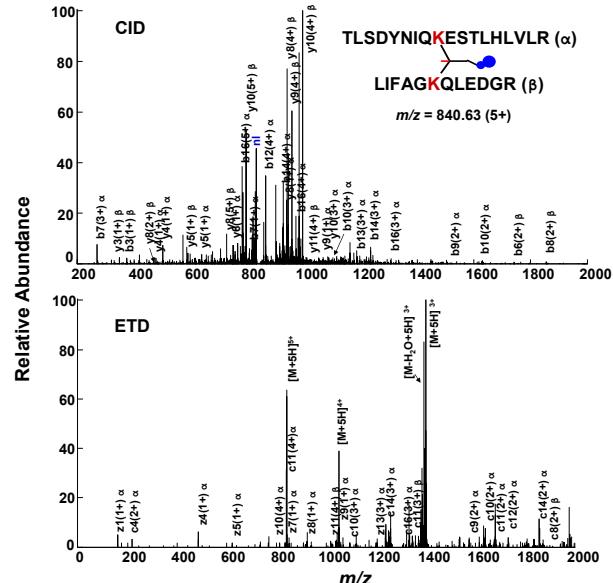


Figure 5: An example of a sequential CID and ETD-MS/MS of an inter cross-linked peptide from Ubiquitin after enrichment. nl: neutral loss of the nitro group.

### Comparison of cross-linking sites with crystal structure of Ubiquitin

Ubiquitin Protein Sequence:  
MQIFVKLTGKITLEVEPSTDITIENVAKA**IQDK^EGIPPDQQR**  
**LIFAGK^QLEDGR** TLS(DYNIQ^KESTLHLVLR)GG

#### Inter-cross-linked peptides

LIFAGK^QLEDGR---TLS(DYNIQ^KESTLHLV LR)

LIFAGK^QLEDGR--- LIFAGK^QLEDGR

LIFAGK^QLEDGR--- IQDK^EGIPPDQQR

#### Intra-cross-linked peptides

AK^IQDK^EGIPPDQQR

#### Dead-end peptides

LIFAGK^QLEDGR

TLS(DYNIQ^KESTLHLV LR)

Figure 6: Ubiquitin sequence, crystal structure (pdb:1V80) and color coded cross-linking sites identified by using CLIP. Identified cross-linked peptides matched the distance constraints of cross-linked lysines and CLIP (~20Å).

## Conclusions

- We demonstrated a novel compact cross-linker with robust enrichment functionality.
- Enrichment has been demonstrated in high complexity cell lysate background.
- Confident cross-linked peptides identification were demonstrated before and after enrichment.
- Sequential application of CID and ETD-MS/MS has been demonstrated for inter-cross-linked peptides for unambiguous identifications.
- This cross-linker and associated analysis strategy are a step towards developing a mass spectrometry oriented global approach to study protein-protein interactions.

## Acknowledgements

This work was funded by LDRD in PNNL, NIH (NIAID). Samples were analyzed using capabilities developed under the support of the NIH National Center for Research Resources (RR18522) and the U.S. Department of Energy Biological and Environmental Research (DOE/BER).

Significant portions of the work were performed in the Environmental Molecular Science Laboratory, a DOE/BER national scientific user facility at Pacific Northwest National Laboratory (PNNL) in Richland, Washington. PNNL is operated for the DOE by Battelle under contract DE-AC05-76RLO-1830.

**CONTACT:** Saiful M. Chowdhury, Ph.D.  
Biological Sciences Division, K8-98  
Pacific Northwest National Laboratory  
P.O. Box 999, Richland, WA 99352  
E-mail: saiful.chowdhury@pnl.gov