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Acetylation of lysines on affinity-purification matrices to reduce co-digestion of bead-bound ligands V.1

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In mass-spectrometry-based interaction proteomics on-bead digestion protocols are commonly applied after affinity-enrichment due to their simplicity and high efficiency. However, on-bead digestion often leads to strong background signals due to co-digestion of the bead-bound ligands such as streptavidin or antibodies. We present an effective, rapid and low-cost method to specifically reduce the peptide signals from co-digested matrix ligands. A short pre-incubation of matrix beads with Sulfo-NHS-Acetate (S-NHS-Ac) leads to acetylation of free amines on lysine side-chains of the bead-bound ligands making them resistant to Lys-C-mediated proteolysis. After binding of bait proteins to the acetylated beads we employ a two-step digestion protocol with the sequential use of Lys-C protease for on-bead digestion followed by in-solution digestion with trypsin. The strong reduction of interfering ligand peptides improves signal strength and data quality for the peptides of interest in liquid chromatography mass spectrometry (LC-MS).

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protocol

Holzer E, Rumpf-Kienzl C, Falk S, Dammermann A (2022) A modified TurboID approach identifies tissue-specific centriolar components in *C. elegans*. PLoS Genet 18(4): e1010150. doi: 10.1371/journal.pgen.1010150

on-bead digestion, affinity-purification, proteomics, BioID, streptavidin, acetylation, TurboID, interaction proteomics, LC-MS

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This protocol has been successfully tested for:
Pierce™ Streptavidin Magnetic Beads **Thermo Fisher Catalog #88816** and GFP-Trap Magnetic Agarose **ChromoTek Catalog #gtma-100**
Application to other affinity matrices or antibodies requires prior testing to ensure that the binding efficiency is not affected.

Please note that Rafiee et al. (https://doi.org/10.15252/msb.20199370) have described a similar approach for the modification of lysines and arginines using a different chemistry for bead modfication.

Acetylation protocol:

Buffers:

- *Reaction buffer:* 50 mM HEPES-NaOH, pH 7.8, 0.2 % Tween 20; you will require at least 17x the desired bead suspension volume (e.g. 340 µL for 20 µL beads, incl. 1 volume shrink).

- *Quenching buffer:* 50 mM Ammonium bicarbonate (=Ammonium hydrogen carbonate), 0.2% Tween 20; you will require at least 16x the desired bead suspension volume (e.g. 320 µL for 20 µL beads, incl. 1 volume shrink).

- *Storage buffer:* 1x PBS-T (i.e. 1x PBS, pH 7.4, 0.2 % Tween 20), 0.02 % Sodium azide; you will require at least 6x the desired bead volume (e.g. 120 µL for 20 µL beads, incl. 1 volume shrink).

Reagents:

Pierce™ Sulfo-NHS-Acetate **Thermo**

- **Fisher Catalog #26777** ; prepare a 100 mM stock solution in water-free DMSO, aliquot and store at -80°C; note that the reagent is labile in water.

Beads as required (see guidelines):

Pierce™ Streptavidin Magnetic Beads **Thermo**

- **Fisher Catalog #88816**

or

GFP-Trap Magnetic

- Agarose **ChromoTek Catalog #gtma-100**

Further materials required:

- Magnetic rack suitable for the applied tube size.

On-bead digestion protocol:

Buffers & reagents

NOTE: Use ultra-pure water for the preparation of all solutions.

- *ABC buffer*: 50 mM Ammonium bicarbonate (=Ammonium hydrogen carbonate; purity ≥ 99.5%), prepare fresh (min. 300 µL per sample, depending on bead volume)

- 1 M urea (≥ 99.5%) in ABC buffer, prepare fresh, do not heat (min. 50 µL per sample, depending on bead volume used)

- *DTT stock solution*: 250 mM dithiothreitol (97%) in water (stock solution aliquots can be prepared before and kept frozen until use; min. 4 µL)

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- *IAA stock solution*: 500 mM iodoacetamide (purity ≥ 99.5%) in water (prepare fresh, min. 4 µL per sample required, depending on set-up)
- *Lys-C solution*: 100 ng/µL lysyl-endopeptidase C (Mass spectrometry grade) in water (stock solution aliquots can be prepared before and kept frozen until use; 150 ng per sample required, depending on set-up and protein amount)
- *Trypsin solution*: 100 ng/µL trypsin (Mass spectrometry grade) in 1 mM HCl (stock solution aliquots can be prepared before and kept frozen until use; 150 ng per sample required, depending on set-up and protein amount)
- 10% trifluoroacetic acid (sequencing grade; TFA)

Further materials required:

- Low-protein-binding PCR tubes
- Magnetic rack for PCR tubes

Please refer to the safety-data-sheets of the according chemicals, especially before handling Sodium azide and Sulfo-NHS-acetate.

This protocol consists of two parts: 1. bead acetylation, 2. on-bead digestion after the affinity enrichment experiment. The protocol for the enrichment depends on the experiment and is not part of this protocol. Please note that the preparations below only relate to part 1 (bead acetylation). Preparations necessary for on-bead digestion are mentioned in the according protocol step and in Materials.

Preparations for bead acetylation:

We recommend to prepare one large batch of beads for the same experimental series. The protocol allows the acetylation of 20 µL beads and can be simply scaled to the amounts required. Please determine and prepare the required reagents accordingly before starting the protocol.

When using magnetic beads all washing steps are performed using a magnetic rack to allow easy removal of buffer.

Using storage buffer is not necessary when the beads are used for affinity purification directly after washing. For longer storage it is highly recommended to prevent bacterial growth.

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Acetylation protocol 1h 30m

1 Wash beads 3x with 100µl *Reaction buffer*, using the magnetic rack. Remove supernatant after the final wash. 10m

2 ⚠ 1h 10m

Add 19 µl *Reaction buffer* and 1 µl Sulfo-NHS-Acetate (100 mM) to obtain a final concentration of 5 mM Sulfo-NHS-Acetate.
Incubate 1h at room temperature with gentle mixing.

NOTE: for up to 20 µL bead suspension use 20 µL reaction volume. For higher amounts use the bead suspension volume as the reaction volume, keeping the Sulfo-NHS-Acetate concentration at 5 mM. Consider that the reagent is labile in water. Thus the buffer must not be prepared before but the reagent must be added from stock immediately to the buffer.

3 Discard supernatant and wash 3x with 100 µl *Quenching buffer*. 10m

4 Use directly or store in 100 µL *Storage buffer* at 4°C for later use.

Affinity enrichment protocol

5 This step is not part of the protocol and will depend on the experiment, as for example BioID, enrichment via GFP and GFP-nanobodies, etc.

NOTE: Be aware that detergents as for example Triton X100 or Tween 20, can be used during these steps but should be removed by repeated washing with detergent-free buffer. Even small amounts of detergent can strongly interfere with chromatography and mass spectrometry and thus should be avoided by all means. We usually recommend five washes with a detergent free buffer before continuing to the on-bead digest protocol. Alternatively, clean-up protocols that are capable of reliably removing detergents could be used after digestion.

On-bead digest protocol 1d 1h 10m

6 Prepare all reagents and materials as specified in Materials. 30m

7 Remove remaining wash buffer from beads. Add 150 µL ABC buffer, gently mix and transfer 5m

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the beads to a 0.2 mL PCR tube and discard supernatant.

NOTE: The volumes specified in the following steps work depend on initial bead volumes and thus might have to be adjusted accordingly. The volumes specified in this protocol work for up to 30 µL GFP-trap magnetic agarose or 200 µL of streptaviding magnetic beads (see Materials).

8 5m

Add 50 µL of *Digestion buffer*.

9 35m

Add 2 µL *DTT stock solution* to reach a final concentration of 10 mM. Incubate for 30 min at room temperature.

10 35m

Add 2 µL *IAA stock solution* to reach a final concentration of 20 mM. Incubate for 30 min at room temperature in the dark.

11 15m

Quench remaining reactive IAA by adding 1 µL *DTT stock solution* (half the amount used in step 8) and incubate for 10 min at room temperature in the dark.

12 18h

Add 1.5 µL (150 ng) *Lys-C solution* and digest over night at 25°C in the dark.

13 5h

Transfer supernatant to a new PCR tube. Add 1.5 µL (150 ng) *Trypsin solution* and digest at 37°C for 5h.

14 5m

Acidify sample by adding 3 µL 10% *TFA* to reach a final concentration of approximately 0.5%.

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Proceed to desalting using according protocols (e.g. C18 StageTips or similar).

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