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David M. Hollenstein^{1,2}, Margarita Maurer-Granofszky³, Dorothea Anrather¹, Thomas Gossenreiter¹, Natascha Hartl¹, Markus Hartl^{1,2}

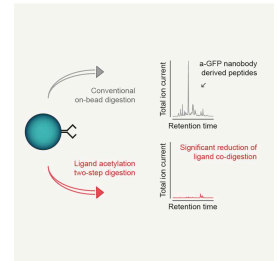
¹Max Perutz Labs, Mass Spectrometry Facility, Vienna Biocenter Campus (VBC), Dr. Bohr-Gasse 9, Vienna, Austria;

²University of Vienna, Center for Molecular Biology, Department for Biochemistry and Cell Biology, Dr. Bohr-Gasse 9, 1030, Vienna, Austria;

³St. Anna Children's Cancer Research Institute (CCRI), Vienna, Austria

**Markus Hartl**

Max Perutz Labs, Mass Spectrometry Facility, Vienna Biocente...



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Chemical Acetylation of Ligands and Two-Step Digestion Protocol for Reducing Codigestion in Affinity Purification–Mass Spectrometry

David M. Hollenstein, Margarita Maurer-Granofszky, Wolfgang Reiter, Dorothea Anrather, Thomas Gossenreiter, Riccardo Babic, Natascha Hartl, Claudine Kraft, and Markus Hartl

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We use this protocol and it's working

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Abstract

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).




Guidelines

This protocol has been successfully tested for:

 Pierce™ Streptavidin Magnetic Beads **Thermo Fisher Scientific Catalog #88816**

and

 ChromoTek GFP-Trap® Magnetic Agarose **Proteintech Catalog #gtma**

Application to other affinity matrices or antibodies requires prior testing to ensure that the binding efficiency is not affected.




Materials

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 μ L for 20 μ L 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 μ L for 20 μ L 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 μ L for 20 μ L beads, incl. 1 volume shrink).


Reagents:

-  Pierce™ Sulfo-NHS-Acetate Thermo Fisher Scientific 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 Scientific Catalog #88816

or

-  ChromoTek GFP-Trap® Magnetic Agarose Proteintech Catalog #gtma

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 $\geq 99.5\%$), prepare fresh (min. 300 μL per sample, depending on bead volume)
- 1 M urea ($\geq 99.5\%$) 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 per sample depending on volumes used)
- *IAA stock solution*: 500 mM iodoacetamide (purity $\geq 99.5\%$) in water (prepare fresh, min. 4 μL per sample required, depending on volumes used)
- *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

Troubleshooting

Safety warnings

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



Before start

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 μ L 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.



Acetylation protocol

1h 30m

- 1 Wash beads 3x with 100µl *Reaction buffer*, using the magnetic rack. Remove supernatant after the final wash. 10m
- 2 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. 1h 10m 
- 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 the beads to a 0.2 mL PCR tube and discard supernatant. 5m

**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 streptavidin magnetic beads (see Materials).

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|----|---|-----|
| 8 | Add 50 μL of 1 M urea in <i>ABC buffer</i> . | 5m |
| 9 | Add 2 μL <i>DTT stock solution</i> to reach a final concentration of 10 mM. Incubate for 30 min at room temperature. | 35m |
| 10 | Add 2 μL <i>IAA stock solution</i> to reach a final concentration of 20 mM. Incubate for 30 min at room temperature in the dark. | 35m |
| 11 | Quench remaining reactive IAA by adding 1 μL <i>DTT stock solution</i> (half the amount used in step 9) and incubate for 10 min at room temperature in the dark. | 15m |
| 12 | Add 1.5 μL (150 ng) <i>Lys-C solution</i> and digest over night at 25°C in the dark. | 18h |
| 13 | Transfer supernatant to a new PCR tube. Add 1.5 μL (150 ng) <i>Trypsin solution</i> and digest at 37°C for 5h. | 5h |
| 14 | Acidify sample by adding 3 μL 10% <i>TFA</i> to reach a final concentration of approximately 0.5%. | 5m |
| 15 | Proceed to desalting using according protocols (e.g. C18 StageTips or similar). | |