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Biotinylation by antibody recognition

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ABSTRACT

This protocol details the biotinylation by antibody recognition.

ATTACHMENTS

956-2485.docx





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Protocol status: Working We use this protocol and it's working

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MATERIALS

PROTOCOL integer ID: 94351

Crosslink reversal buffer

Funders Acknowledgement:

NCI

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NINDS

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	A	В
	SDS	5%
	Tris-HCl pH 8.0	500 mM
Γ	NaCl	150 mM
	EDTA	2 mM

Modified TBST

A	В
Tris-HCl	20 mM
NaCl	200 mM
EDTA	2 mM
Triton X-100	0.5%

Stringent wash buffer

А	В
Tris-HCl pH 7.6	20 mM
NaCl	200 mM
SDS	0.1%
EDTA	2 mM

TBST

A	В
Tris-HCl pH 7.6	20 mM
NaCl	150 mM
Tween-20	0.1%

High stringency wash buffer

А	В
Tris-HCl pH 7.6	20 mM

	A	В
Г	NaCl	400 mM
	Tween-20	0.1%

⊠ Trypsin **Promega Catalog #V5111**

⊠ Clarity Western ECL Substrate **Bio-Rad Laboratories Catalog #1705060**

7h 45m Biotinylation by antibody recognition 1 1h Collect the brain sections at 240-micron intervals across the neuroaxis, place them into a net well (Brain research laboratories) and wash 3 times for (5) 01:00:00 each in TBST. 2 1h Place the sections in 0.3% hydrogen peroxide and 0.1% sodium azide diluted in blocking buffer for 01:00:00 at \$\mathbb{s}\$ Room temperature to quench endogenous peroxidases. 3 Rinse the sections briefly in TBST and incubate in anti-PSER129 antibody EP1536Y diluted 1:50,000 in blocking buffer Overnight at 4 °C with gentle agitation. 4 The following day, wash the sections 3 times in TBST, then incubate with biotinylated anti-rabbit antibody diluted 1:200 in blocking buffer for 60 01:00:00 at 8 Room temperature Wash the sections 3 times in TBST, incubate with ABC reagent for 01:00:00, and wash off with borate 1h buffer.



6 Incubate the sections with borate buffer containing biotinyl tyramide as described above.





Briefly sonicate each sample in 1 mL of crosslink reversal buffer (refer materials section) and hea 1h 30m



9 Centrifuge the sample 20000 x g, 00:20:00 of the samples and then dilute the supernatant 1:10 in modified TBST (refer materials section).



Incubate each sample with 40 mg of streptavidin magnetic beads (Thermofisher Scientific) for 02:00:00 at Room temperature with constant mixing.

2h



11

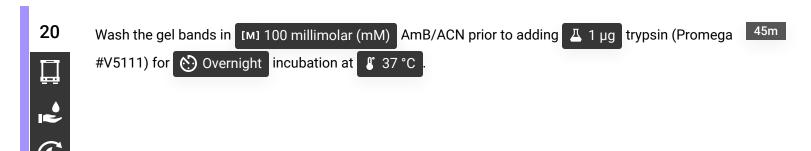
Collect the beads using a magnetic stand (Thermofisher Scientific), wash the beads 3 times in modified TBST, and then Overnight in 10 mL of stringent wash buffer (refer materials section).





- The following day, collect the beads using magnetic stand and resuspend in $2 100 \,\mu$ L 1 X Bolt LDS sample buffer with reducing agent (Thermofisher) then heat for 0 00:10:00 at $98 \,^{\circ}$ C.
- Vortex the samples vigorously and remove the beads using magnetic stand.
- Subject Δ 70 μL of the sample to electrophoresis approximately 2 cm into a Bolt gel (ThermoFisher).
- Fix the gel in 50% ethanol and 10% acetic acid for 01:00:00
- 16 Wash the gel several times in dH20, and stain the proteins with colloidal Coomassie blue.
- 17 Then excise the entire sample for trypsin digestion and mass spectrometry.
- 9
- Wash the gel pieces with [M] 100 millimolar (mM) ammonium bicarbonate (AmB)/acetonitrile (ACN) and 45m
- Alkylate the cysteines using [M] 100 millimolar (mM) iodoacetamide in the dark for 00:45:00 at Room temperature (RT).

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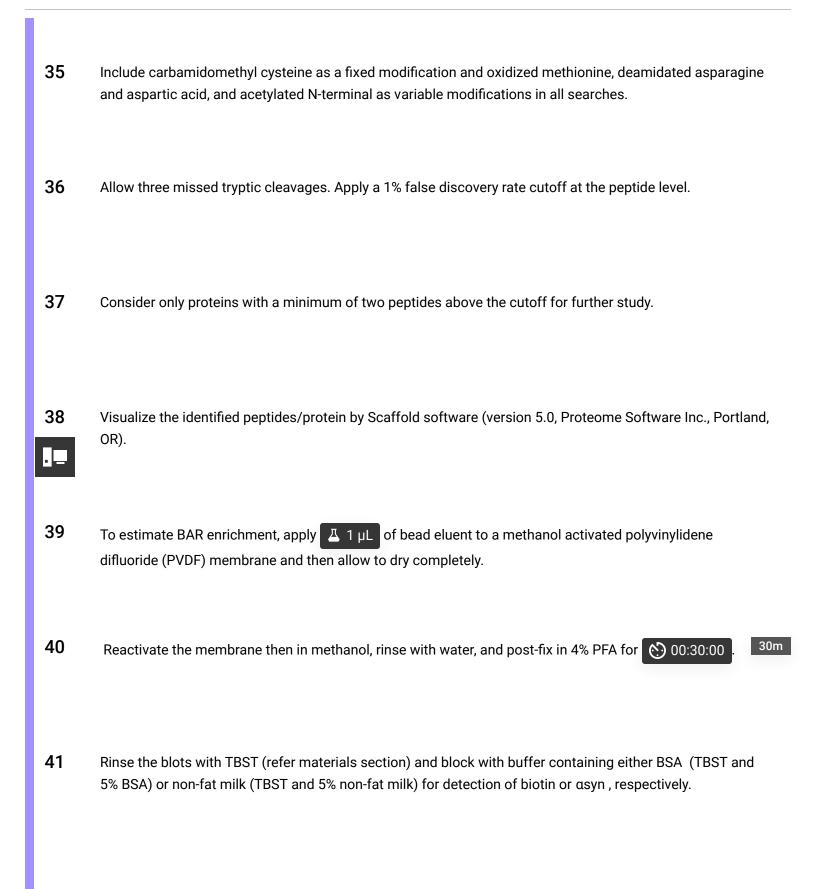


- 21 Collect the peptide containing supernatants into a separate tube.
- Wash the gel pieces with gentle shaking in 50% ACN/1% FA at Room temperature for 00:10:00, and collect the supernatant in the previous tubes.
- Do the final peptide extraction step with 80% ACN/1% FA, and 100% ACN, and collect all supernatant.
- Dry the peptides in a speedvac and reconstitute with 5% ACN/0.1% FA in water before injecting into LC-MS/MS.
- Analyse the peptides by LC-MS/MS using a Dionex UltiMate 3000 Rapid Separation nanoLC coupled to an Orbitrap Elite Mass Spectrometer (Thermo Fisher Scientific Inc.).
- Load the samples onto the trap column, which is 150 μ m x 3 cm in-house packed with 3 μ m ReproSil-Pur® beads.

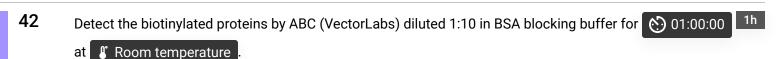
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- The analytical column is a 75 μm x 10.5 cm PicoChip column packed with 3 μm ReproSil-Pur® beads (New Objective, Inc. Woburn, MA).
- 28 Keep the flow rate at 300 nL/min.
- Ellute all the fractions from the analytical column at a flow rate of 300 nL/min using an initial gradient elution of 5% B from 00:00:00 to 00:05:00, transition to 40% over 00:40:00, 60% for 00:04:00, ramping up to 90% B for 00:03:00, holding 90% B for 00:03:00, followed by reequilibration of 5% B at 00:10:00 with a total run time of 00:00:00.
- Record the mass spectra (MS) and tandem mass spectra (MS/MS) in positive-ion and high-sensitivity mode with a resolution of ~60,000 full-width half-maximum.
- 31 Select the 15 most abundant precursor ions in each MS1 scan for fragmentation by collision-induced dissociation (CID) at 35% normalized collision energy in the ion trap.
- Dynamically excluded the previously selected ions from re-selection for © 00:01:00. Store the collected raw files spectra in. raw format.
- Identify the proteins from the MS raw files using the Mascot search engine (Matrix Science, London, UK. version 2.5.1).
- 34 Search the MS/MS spectra against the SwissProt mouse database.

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- 43 Asyn can be detected using SYN1 (BD Biosciences) diluted 1:2,000 and PSER129 detected using EP1536Y diluted 1:50,000 both diluted in non-fat milk blocking buffer.
- Detect the primary antibodies by incubating blots for 01:00:00 in secondary anti-mouse HRP conjugat the diluted 1:6,000 or secondary anti-rabbit HRP conjugate (Cell signaling) diluted in milk blocking buffer.
- Following secondary antibody, wash the membranes in high stringency wash buffer (Refer materials section) and image using enhanced chemiluminescence (ECL) substrate (Biorad, product # 1705060) and Chemidoc imager (Biorad).