JAN 29, 2024

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DOI:
dx.doi.org/10.17504/protocols.io.14egn3qq6l5d/v1

Protocol Citation: Bryan_Killinger 2024. Biotinylation by antibody recognition. **protocols.io** https://dx.doi.org/10.17504/protocols.io.14egn3qq6l5d/v1

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

Created: Jan 16, 2024

Biotinylation by antibody recognition

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ABSTRACT

This protocol details the biotinylation by antibody recognition.

ATTACHMENTS

956-2485.docx

Last Modified: Jan 29, 2024

PROTOCOL integer ID: 94351

Funders Acknowledgement:
NCI
Grant ID: CCSG P30 CA060553
NIH Office of Director
Grant ID: S10OD025194
National Resource for Translational and Developmental Proteomics
Grant ID: P41 GM108569
EraPerMed DEEPEN-iRBD project
Grant ID: ANR-22-PERM-0006
Michael J. Fox Foundation
Grant ID: ASAP-000458
NINDS
Grant ID: 1R01NS128467
Michael J. Fox Foundation
Grant ID: ASAP-021030
NIH
Grant ID: R21 NS109871
NINDS
Grant ID: K23-NS097625-06

MATERIALS

Crosslink reversal buffer

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

Modified TBST

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

Stringent wash buffer

	A	B
	Tris-HCl pH 7.6	20 mM
	NaCl	200 mM
	SDS	0.1%
	EDTA	2 mM

TBST

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

High stringency wash buffer

	A	B
	Tris-HCl pH 7.6	20 mM

	A	B
	NaCl	400 mM
	Tween-20	0.1%

Trypsin **Promega Catalog #V5111**

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

Biotinylation by antibody recognition

7h 45m

1 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 01:00:00 each in TBST. 1h

2 Place the sections in 0.3% hydrogen peroxide and 0.1% sodium azide diluted in blocking buffer for 01:00:00 at Room temperature to quench endogenous peroxidases. 1h

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. 1h

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 01:00:00 at Room temperature 1h

5 Wash the sections 3 times in TBST, incubate with ABC reagent for 01:00:00, and wash off with borate buffer. 1h

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

7 Wash the sections Overnight with TBST, gather in a 1.5mL Eppendorf tube, 3000 x g, 00:15:0(1h 15m pellet floating sections, and discard the supernatant.

8 Briefly sonicate each sample in 1 mL of crosslink reversal buffer (refer materials section) and hea 1h 30m 00:30:00 at 98 °C followed by 01:00:00 at 90 °C .

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

10 Incubate each sample with 40 mg of streptavidin magnetic beads (Thermofisher Scientific) for 2h 02:00:00 at Room temperature with constant mixing.

11 Collect the beads using a magnetic stand (Thermofisher Scientific), wash the beads 3 times in modified 2h TBST, and then Overnight in 10 mL of stringent wash buffer (refer materials section).

12 The following day, collect the beads using magnetic stand and resuspend in 100 µL 1 X Bolt LDS sample buffer with reducing agent (Thermofisher) then heat for 00:10:00 at 98 °C. 10m

13 Vortex the samples vigorously and remove the beads using magnetic stand.

14 Subject 70 µL of the sample to electrophoresis approximately 2 cm into a Bolt gel (ThermoFisher).

15 Fix the gel in 50% ethanol and 10% acetic acid for 01:00:00. 1h

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.

18 Wash the gel pieces with [M] 100 millimolar (mM) ammonium bicarbonate (AmB)/acetonitrile (ACN) and reduce with [M] 10 millimolar (mM) dithiothreitol (DTT) at 50 °C for 00:45:00. 45m

19 Alkylate the cysteines using [M] 100 millimolar (mM) iodoacetamide in the dark for 00:45:00 at Room temperature (RT). 45m

20 Wash the gel bands in [M] 100 millimolar (mM) AmB/ACN prior to adding 1 µg trypsin (Promega #V5111) for Overnight incubation at 37 °C. 45m

21 Collect the peptide containing supernatants into a separate tube.

22 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. 10m

23 Do the final peptide extraction step with 80% ACN/1% FA, and 100% ACN, and collect all supernatant.

24 Dry the peptides in a speedvac and reconstitute with 5% ACN/0.1% FA in water before injecting into LC-MS/MS.

25 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.).

26 Load the samples onto the trap column, which is 150 µm x 3 cm in-house packed with 3 µm ReproSil-Pur® beads.

27 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.

29 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 01:40:00, 60% for 00:04:00, ramping up to 90% B for 00:03:00, holding 90% B for 00:03:00, followed by re-equilibration of 5% B at 00:10:00 with a total run time of 02:00:00. 4h 5m

30 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.

32 Dynamically excluded the previously selected ions from re-selection for 00:01:00. Store the collected raw files spectra in. raw format. 1m

33 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.

35 Include carbamidomethyl cysteine as a fixed modification and oxidized methionine, deamidated asparagine and aspartic acid, and acetylated N-terminal as variable modifications in all searches.

36 Allow three missed tryptic cleavages. Apply a 1% false discovery rate cutoff at the peptide level.

37 Consider only proteins with a minimum of two peptides above the cutoff for further study.

38 Visualize the identified peptides/protein by Scaffold software (version 5.0, Proteome Software Inc., Portland, OR).

39 To estimate BAR enrichment, apply 1 µL of bead eluent to a methanol activated polyvinylidene difluoride (PVDF) membrane and then allow to dry completely.

40 Reactivate the membrane then in methanol, rinse with water, and post-fix in 4% PFA for 00:30:00 . 30m

41 Rinse the blots with TBST (refer materials section) and block with buffer containing either BSA (TBST and 5% BSA) or non-fat milk (TBST and 5% non-fat milk) for detection of biotin or αsyn , respectively.

42 Detect the biotinylated proteins by ABC (VectorLabs) diluted 1:10 in BSA blocking buffer for 01:00:00 1h at Room temperature.

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.

44 Detect the primary antibodies by incubating blots for 01:00:00 1h in secondary anti-mouse HRP conjugat diluted 1:6,000 or secondary anti-rabbit HRP conjugate (Cell signaling) diluted in milk blocking buffer.

45 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).