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APEX2 labelling and screening for biotinylated proteins by proteomics

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Protocol status: Working

We use this protocol and it's working

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Disclaimer

Protocol under optimization

Abstract

Enzyme-catalyzed proximity labeling (PL) combined with mass spectrometry (MS) has emerged as a revolutionary approach to reveal the subcellular proteomes. APEX (engineered ascorbate peroxidase) catalyzes the oxidation of biotin-phenol to the short-lived biotin-phenoxyl radical in the presence of hydrogen peroxide. This radical reacts with electron-rich amino acids such as tyrosine on neighboring proteins resulting in their biotinylation. APEX2 technology uses a mutant enzyme that increases sensitivity. This protocol describes APEX2 labelling and sample procurement for mass spectrometry from SNc and striatum.



Materials

Cutting Solution

* dilute to 1x add 1x Na bicarbonate, Na ascorbate, MgCl₂ and CaCl₂

-make 200ml per mouse, and store in the -80°C until just frozen

	Compound	final concentration (mM)		
	Choline	110		
	MgCl ₂	7		
	KCl	2.5		
	NaH ₂ PO ₄ *2H ₂ O	1.25		
	CaCl ₂	0.5		
	Glucose	10		
	Na ascorbate	1.3		
	Na bicarbonate	25		

aCSF

-initial incubation in 0.5mM of BP (-20°C) and 1uM TTX

-quenching solution add 10mM Trolox, 20mM sodium ascorbate, and 10mM sodium azide

	aCSF			
	Compound	final concentration (mM)		
	KCl	2.5		
	Glucose	10		
	NaCl	125.2		
	NaH ₂ PO ₄ *H ₂ O	0.3		



	MgCl ₂ *6H ₂ O	1.3		
	CaCl ₂ *2H ₂ O	2.4		
	NaHCO ₃	26		

Hydrogen Peroxide

Prepare 1M H₂O₂ in PBS and dilute to a final concentration of 1mM for reaction initiation

*prepare fresh daily

i.510ul of H₂O₂ into 5ml of H₂O 1M solution

ii.700μl of 1M solution into aCSF

Tissue Lysis Buffer

*make roughly 1ml per reaction

	Lysis Buffer	final concentration mM
	Tris	50
	NaCl	150
	EDTA	10
	Triton	1%
	Trolox	5
	Sodium Ascorbate	10
	Sodium Azide	10
	protease inhibitor	1x

TCA Solution

*TCA solution is a 100% solution in glass amber bottle in 4°C. Stable for ~23weeks

-dilute TCA to working concentration of 55%, make fresh daily



Acetone

-store in -20°C 1 day before use

-I got mine from the Decaen lab but they don't have a lot so we might need to consider getting our own.

-Need ~5ml per reaction

Urea Dissolve Buffer

*1ml per sample

		final concentration (M)
	Urea	8
	SDS	1%
	Sodium phosphate Buffer, pH 8	0.1
	ammion bicarbonate	0.1

*Prepare

Sodium phosphate buffer by mixing 1M NaH₂PO₄ (monobasic) and 1 M Na₂HPO₄ (dibasic)

<http://cshprotocols.cshlp.org/content/2006/1/pdb.rec8303>

Urea Detergent Wash Buffer

*~3ml per sample Day1, and ~2ml per sample Day 2

	Urea Detergent Wash Buffer	final concentration (M)	
	Urea		4
	SDS		0.50%



	sodium phosphate buffer	0.1	

Urea Wash Buffer (NO SDS)

*~2ml per sample

	Urea Wash Buffer		final concentration (M)
	Urea		4
	sodium phosphate buffer	0.1	

APEX2 Labelling

- 1 Construct: AAV5-CAG-DIO-APEX2NES is injected into the ventral midbrain (VM) of DATIRES-Cre mice
- 2 Confirmation of cellular specificity: Representative slices of SN and Str stained for TH, V5 and merged
- 3 Brains perfused with aCSF by transcardial perfusion with 10ml ice cold cutting solution
- 4 Acute slice preparation as 300µm coronal slices
- 5 Incubation of slices with 0.5mM BP in oxygenated artificial cerebrospinal fluid (aCSF) during the slice recovery period (1hr RT)
- 6 Rapid biotinylation: rapid labeling with 1 mM H₂O₂ in aCSF for 3 minutes
- 7 Rapid quenching by transferring slices to quenching aCSF (20mM Sodium ascorbate, 10mM NaN₃ and 10mM Trolox) for 5 minutes.
- 8 Slices were transferred to ice-cold quenching aCSF for rapid dissection, tissue were flash frozen and stored in -80C for downstream western blot or proteomics. Tissue lysis and capture biotinylated proteins for proteomics

Tissue lysis and capture of biotinylated proteins for proteomics

- 9 Frozen tissues or synaptosome pellets were homogenized on ice in a glass dounce homogenizer with 30 strokes (in 750ul lysis buffer with protease and phosphatase inhibitor). One mouse/sample for striatum and 2mice/sample for SNc.
- 10 Lysates with addition 39ul of 10% SDS were rotated for 15 min at 4°C
- 11 Lysates were clarified by centrifugation at 21,000 × g for 10 min at 4°C

- 12 Supernatants were transferred to a new prechilled Eppendorf tube for trichloroacetic acid (TCA) precipitation (for MS)
- 13 Proteins were precipitated from lysates by the addition of an equal volume of ice-cold 55% TCA.
- 14 Samples were incubated on ice for 15 min, followed by centrifugation at $21,000 \times g$ for 10 min at 4°C .
- 15 Protein pellets were resuspended in 1 ml of acetone prechilled to -20°C and recentrifuged. Pellets were resuspended and recentrifuged another three times in 1 ml of acetone prechilled to -20°C , for a total of four washes
- 16 Protein pellets were resuspended in Urea Dissolve Buffer (with protease and phosphatase inhibitor) and sonicate for 10 second followed by gentle agitation on an orbital shaker for 1 hr at room temperature.
- 17 Streptavidin magnetic beads (Thermo Fisher #88817) were resuspended and washed three times in Urea Detergent Wash Buffer for 10 min at 4°C . After washing, streptavidin beads were resuspended in ice-cold Urea Detergent Wash Buffer and 50 μl containing 0.5 mg of beads was added to each sample.
- 18 Proteins were incubated with streptavidin beads overnight on a rotor at 4°C for 14-18hrs.
- 19 Beads were washed three times for 5-10 min in 1 ml of Urea Detergent Wash Buffer at room temperature. After the third wash, beads were resuspended in 1 ml of Urea Wash Buffer and transferred to a new tube
- 20 After three 5-10 min washes in 1ml Urea Wash Buffer at room temperature, beads were resuspended in 200 μl of Urea Wash Buffer and transferred to a new tube
- 21 10 μl aliquot (5%) was transferred to a separate tube for western blotting, and the remaining 190 μl of buffer were removed on a magnetic stand. Beads were flash frozen and stored at -80°C