Alzforum Protocol Submission Form

Date Submitted: Aug. 20, 2007
Protocol Title: Immunostaining for neprilysin
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_X_ This submission has been approved by Lab PI

Categories: Place an X next to all that apply to this protocol.
Please add a new category name if needed.

Proteins		**Methods**		**Species**	
All (default)		All (default)		All (default)	
Abeta		Protein-Enzyme Assay		Rodent	X
Tau		Tissue Preparation		Human	
Neprilysin	X	Cell Culture		Other	
ACE		Immunohistochemistry	X		
		Molecular Biology			
		Elisa			
		Western Blotting			
		Protein Isolation/Purification			
		Gene Expression			
		Collection of Biofluids			

Overview (Text that describes the purpose of the protocol)

This is a protocol for the immunostaining of neprilysin using the high temperature antigen unmasking technique and the immunoperoxidase-indirect or fluorescence-indirect tyramide signal amplification (TSA) method. Among various methods for antigen retrieval, including autoclaving, microwaving, formic acid or detergent treatment, and tryptic digestion, autoclaving at 121°C for 5 min gives the best results. Without autoclaving, no immunoreactivity is detected. Signals detected by this protocol are highly specific for neprilysin, because no signal is detected in the brain of neprilysin-deficient mice. For fixation of tissue specimen, 4% paraformaldehyde is preferable. A paraffin section is much better than frozen sections because of a procedure for autoclaving.

Reagents (List of reagents used in the protocol; include company and catalog number for antibodies so that we can link to our Antibody database)

- Primary antibody (anti-neprilysin): clone 56C6 (NCL-CD10-270, 1ml, Novocastra Lab.)
- Avidin /biotin blocking kit (SP-2001, Vector Lab.)
- Second antibody: Biotin conjugated-goat anti-mouse IgG_1 (1070-08, 1mg/ml, Southern Biotech.)
- TSA Biotin System (TSA-Indirect kit) (NEL-700A, NEN)
- Alexa Fluor 488-conjugated streptavidin (S-11223, Molecular Probe)
- Perma Fluor Aqueous Mountant (434990, Immunon, Pittsburgh, PA)
- 10mM Sodium citrate buffer, pH6.0.
- 0.3% H_2O_2 in methanol solution
- TN buffer: 0.1M Tris-HCl, 0.15M NaCl, pH 7.5.
- TNT wash buffer: 0.1M Tris-HCl, 0.15M NaCl, 0.05% Tween 20, pH 7.5.
- TNB (blocking buffer): 0.1M Tris-HCl, 0.15M NaCl, 0.5% blocking reagent, pH 7.5.
- ImmunoPure Metal Enhanced DAB substrate (#1850090, PIERCE)

Equipment (List of equipment needed for this protocol)

Confocal laser microscope/Fluorescence microscope

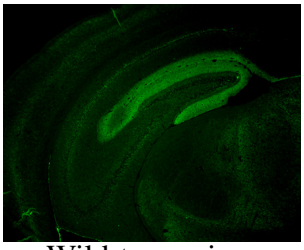
Protocol Procedure (Text that lists steps of protocol; may include images or video)

Immunofluorescence staining

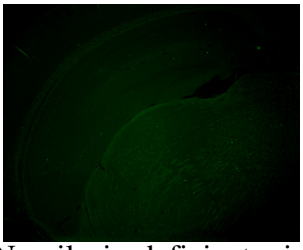
See the manufacturer's instructions for TSA biotin system and avidin/biotin blocking kit before starting!

1	Deparaffinize & rehydrate.	
2	Autoclave in 10mM Sodium citrate buffer (pH6.0) at 121°C. (for epitope retrieval of antigen) *Don't cap a stainless steel cooker containing slides!*	5 min
3	Rinse sections in tap water.	5 min
4	0.3% H_2O_2 in methanol	30 min
5	Rinse in tap water.	5 min
6	Wash in TN.	5 min x 3
7	Avidin blocking (Vector kit)	15 min
8	Biotin blocking (Vector kit)	15 min
9	TSA kit blocking	30 mim
10	Primary antibody (x100-x200, diluted with TNB)	4 °C o/n

11	Wash in TNT.	5 min x 3
12	Biotinylated second antibody (x2,000-3,000, diluted with TNB)	1hr
13	Wash in TNT.	5 min x 3
14	Streptavidin-HRP (x100) (TSA-Indirect kit)	30 min
15	Wash in TNT.	5 min x 3
16	Biotinyl tyramide amplification reagent (x50) (TSA-Indirect kit)	10 min
17	Wash in TNT	5 min x 3
18	Streptavidin-Alexa488 (x500)	30 min
19	Coverslip with Perma Fluor Aqueous Mountant to retard fading.	
20	Capture images with a microscope incorporating a confocal laser scanning system.	



Wild-type mice



Neprilysin-deficient mice

Peroxidase-DAB(Diaminobenzidine)- staining

See the PIERCE's instructions for metal enhanced DAB substrate.

1-17	the same as above	
18'	Streptavidin-HRP (x100) (TSA-Indirect kit)	30 min
19'	Wash in TNT.	5 min x 3
20'	DAB	3 min

References (List up to 5 key citations that describe applications of the protocol]

1. Fukami S, Watanabe K, Iwata N, Haraoka J, Lu B, Gerard NP, Gerard C, Fraser P, Westaway D, St George-Hyslop P, Saido TC. (2002) Aβ-degrading endopeptidase, neprilysin, in mouse brain: synaptic and axonal localization inversely correlating with Aβ pathology. Neurosci Res. 43(1):39-56.

2. Iwata N, Takaki Y, Fukami S, Tsubuki S, Saido TC. (2002) Region-specific reduction of Aβ-degrading endopeptidase, neprilysin, in mouse hippocampus upon aging. J Neurosci Res. 70(3):493-500

3. Iwata N, Mizukami H, Shirotani K, Takaki Y, Muramatsu S, Lu B, Gerard NP, Gerard C, Ozawa K, Saido TC. (2004) Presynaptic localization of neprilysin contributes to efficient clearance of amyloid-β peptide in mouse brain. J Neurosci. 24(4):991-998.
