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

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Immunoblots

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ABSTRACT

This protocol describes a standard procedure for protein separation and identification of protein of interest.

Protocol overview:

The steps of this protocol is similar for different sources of protein such as cell lysate, cytosol, or membrane samples, and EV samples. However, there are some specific changes depending on protein of interest, endogenous alpha-synuclein, or type of cells, hiPSC dopamine neurons.

MATERIALS

Reagents

	Reagent	Catalog number	Manufacturer
	Gel, 4-20%, 15 wells, WedgeWell	XP04205BOX	Thermo Fisher Scientific
	Gel, 4-20% Criterion, 26 wells	345-0034	BioRad
	Gel, 4-20%, 10 wells, WedgeWell	XP04200BOX	Thermo Fisher Scientific
	PVDF, Immobilon P	IPVH00010	EMD Millipore
	Tris base	BP152-5	Fisher Scientific
	Glycine	BP381-5	Fisher Scientific

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	Reagent	Catalog number	Manufacturer
	Methanol		
	PageRuler prestained protein ladder	26617	Thermo Scientific
	TBS		
	Triton X100	T8787-250ML	Sigma-Aldrich
	BSA	A3294	Sigma

Antibodies

	Antibodies	Catalog number	Manufacturer	Dilutions
	Mouse monoclonal anti-α-synuclein	Cat# 610787	BD Biosciences	1:500
	Rabbit polyclonal anti-α-synuclein	Cat# 10842-1-AP	Proteintech	1:500
	Rabbit polyclonal anti-DNAJC5	Cat# 144-10489-200	RayBiotech	1:1,000
	Mouse monoclonal anti-alpha tubulin	Cat# ab7291	Abcam	1:2,000
	Mouse monoclonal anti-Alix	Cat# Sc-53540	Santa Cruz Biotechnology	1:1,000
	Rabbit monoclonal anti-CD9	Cat# 13174S	Cell Signaling Technology	1:1,000
	Mouse monoclonal anti-PDI	Cat# ADI-SPA-891-D	Enzo Life Sciences	1:1,000
	Mouse monoclonal anti-CD63	Cat# BDB556019	Thermo Fisher Scientific	1:1,000
	Mouse monoclonal anti-Flotillin-2	Cat# 610383	BD Biosciences	1:1,000
	Mouse monoclonal anti-Transferrin Receptor	Cat# 13-6800	Thermo Fisher Scientific	1:1,000
	Mouse monoclonal anti-GM130	Cat# 610823	BD Biosciences	1:1,000
	Rabbit monoclonal anti-Tom20	Cat# 42406S	Cell Signaling Technology	1:1,000
	Rabbit polyclonal anti-GFP	Cat# NC9589665	Fisher Scientific	1:1,000
	Rabbit polyclonal anti-LC3B	Cat# NB100-2220	Novus Biologicals	1:1,000
	Rabbit monoclonal anti-Citrate Synthase	Cat# 14309S	Cell Signaling Technology	1:1,000
	Rabbit polyclonal anti-Dopamine transporter	Cat# BS-1714R	Bioss Antibodies	1:1,000
	Rabbit monoclonal anti-beta III Tubulin	Cat# ab215037	Abcam	1:1,000

	Antibodies	Catalog number	Manufacturer	Dilutions
	Rabbit polyclonal anti-Tyrosine hydroxylase	Cat# AB152	Millipore	1:1,000
	Chicken polyclonal Microtubule-associated protein 2	Cat# ab92434	Abcam	1:1,000
	Mouse monoclonal anti-FLAG	Cat# F9291	Sigma-Aldrich	1:1,000

Immunoblots

40m

1 Cell lysate, cytosol, or membrane samples, and EV samples were mixed with SDS sample loading buffer. For cell lysate, cytosol or membrane samples, 20 µg proteins were loaded. For EV samples, the maximal amount up to 20uL were loaded.

2 Samples with DNAJC5 and α-syn were heated at 55°C for 10 min to prevent aggregation. Other samples were heated at 95°C for 5 min and separated on SDS-PAGE gels (Novex wedgewell 4%-20% Tris-Glycine mini gels, 200 V for 45 min).

3 Proteins were transferred to PVDF membranes (EMD Millipore, Darmstadt, Germany) in cold room at 0.6 A for 2 h.

4 Blocked membrane with 5% bovine serum albumin in TBST (20 mM Tris pH 7.4, 150 mM NaCl, and 0.1% Tween-20) at Room temperature for 1 h with constant agitation. 01:00:00 1h

5 Incubated membranes with primary antibodies at 4 °C overnight.

6 For immunoblots from hiPSC dopamine neurons, samples in loading buffer were heated to 70 °C for 00:10:00 and blocking was carried out with 5% skimmed milk. 10m

7 For immunoblots of endogenous α-syn in SH-SY5Y cells, PVDF membranes were fixed with 0.4% paraformaldehyde in TBST at room temperature for 00:30:00 30m

8 Blots were washed with TBST for 5 times, each time with 00:05:00 agitation. 5m

9 Incubate membranes with secondary antibodies, anti-rabbit or anti-mouse for 01:00:00 at Room temperature 1h

10 Detection was performed with Supersignal Chemiluminescent substrate and quantified with Fiji/ImageJ.