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

Ubiquitin immunoprecipitation using an anti-ubiquitin nanobody

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ABSTRACT

This protocol describes a method to detect ubiquitination on a protein of interest. This technique relies on immunoprecipitation (IP) of ubiquitinated proteins from a cell lysate through the use of an anti-ubiquitin nanobody coupled to agarose beads. The eluate can be run on SDS-PAGE to determine whether the protein of interest was recovered in the IP and, therefore, ubiquitinated.

ATTACHMENTS

io_Ubiquitin immunoprecipitation using an anti.docx

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MATERIALS

Materials

Cell culture

2-4 50-80% confluent wells of cells growing in a 6-well plate.

Equipment

Equipment	
Bioruptor® Plus sonication device	NAME
Bioruptor®	BRAND
B01020001	SKU
https://www.diagenode.com/en/p/bioruptor-plus-sonication-device	LINK

- CLARIOstar Plus Plate Reader (BMG Labtech) (or equivalent)
- Microcentrifuge

Specialized Reagents

- Ubiquitin Selector **NanoTag Biotechnologies Catalog #N2510**
- MiniSpin Columns (50 units) **NanoTag Biotechnologies Catalog #A1001-L**
- TrypLE™ Express Enzyme (1X), phenol red **Thermo Fisher Catalog #12605036**
- Pierce™ Rapid Gold BCA Protein Assay Kit **Thermo Fisher Catalog #A53225**

Buffers

- Triton Lysis Buffer:

	A	B
	Tris pH 8	20 mM
	NaCl	150 mM
	Triton X-100	1%
	cOmplete, Mini EDTA-free Protease Inhibitor Cocktail (Roche cat. no. 11873580001)	1X
	Freshly-added 20 mM N-ethylmaleimide (2.5 mg/ml; Sigma Aldrich E3876-25G)	2.5 mg/ml
	50 uM PR-619 (from 100 mM DMSO stock; Sigma-Aldrich 662141-25MG)	50 uM
	Benzonase (Max Planck Institute of Biochemistry Core Facility)	7.5 U/ml

cOmplete™ EDTA-free Protease Inhibitor Cocktail **Roche Catalog #11873580001**

N-Ethylmaleimide **Merck MilliporeSigma (Sigma-Aldrich) Catalog #E3876**

DUB Inhibitor V, PR-619 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #662141**

- Triton Wash Buffer:

	A	B
	Tris pH 8	20 mM
	NaCl	150 mM
	EDTA	1 mM
	Triton X-100	1%
	SDS	0.1%
	PR-619	50 uM

- 4X NuPAGE™ LDS Sample Buffer (4X) **Thermo Fisher Scientific Catalog #NP0007** + 5% beta-mercaptoethanol (2-Mercaptoethanol **Merck MilliporeSigma (Sigma-Aldrich) Catalog #M6250** -100ml)
- 10% FBS (Fetal Bovine Serum **Gibco - Thermo Fischer Catalog #10270106**) in 1X PBS (diluted from PBS (10X), pH 7.4 **Thermo Fisher Scientific Catalog #70011051**)
- 1X PBS

Cell collection and lysis

1 Remove the medium from the wells and trypsinize with 500 µL TrypLE Express.

2 Quench the TrypLE Express with 500 µL 10% FBS and move the cells into a centrifuge tube.

3 Pellet cells at 1500 x g for 00:03:00 at 4 °C. 3m

4 Wash cells with 1 mL PBS and pellet again.

5 Resuspend cells in 150 µL Triton Lysis Buffer and incubate On ice for 00:05:00. 5m

6 Sonicate in the BioRuptor for 5 cycles of 00:00:30 on and 00:00:30 off at 4 °C. 1m

7 Clarify the lysate by centrifugation 18000 x g for 00:10:00 4 °C. 10m

8 During the centrifugation, prepare BSA standards according to the Rapid Gold BCA Protein Assay Kit manual and begin equilibrating 50 µL of Ubiquitin pan-selector resin (pipette with a cut p200 tip) in 500 µL

of lysis buffer.

9 Collect supernatant and place into a new tube On ice.

10 Prepare a small 1:10 dilution of samples and perform Rapid Gold BCA Protein Assay according to manufacturer's instructions.

Immunoprecipitation (IP)

1h 7m

11 Dilute samples into Triton Lysis Buffer in two tubes: 1 mg in 500 µL (for IP) and 100 µg in 25 µL (for input).

12 Pellet the beads at 1000 x g for 00:02:00 at 4 °C. 2m

13 Pull off the supernatant, add the 1 mg of lysate to the beads, and incubate with rotation at 4 °C fo 1h 01:00:00.

14 During the incubation denature the input sample with 25 µL 4X NuPAGE LDS Sample Buffer at 95 °C 00:05:00. 5m

15 Pellet the beads at 1000 x g for 00:02:00 at 4 °C. 2m

16 Wash the beads with either 1 mL Triton Wash Buffer.

17 Pellet the beads and repeat for a total of 3 washes.

18 Remove the supernatant and add 100 µL of 2X NuPAGE LDS Sample Buffer.

19 Elute by boiling at 95 °C for 00:05:00. 5m

20 Transfer the beads to a MiniSpin column placed in a tube.

21 Centrifuge 3000 x g for 00:03:00. 3m

22 The eluate has now been collected in the tube and is ready for SDS-PAGE analysis.