Apr 07, 2025 Version 1

Protein extraction, co-immunoprecipitation, and immunoblots V.1

Nature Cancer

DOI
https://dx.doi.org/10.17504/protocols.io.eq2lyx3mpgx9/v1

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DOI: https://dx.doi.org/10.17504/protocols.io.eq2lyx3mpgx9/v1

External link: https://doi.org/10.1038/s43018-025-01003-3

Protocol Citation: Laura Antonucci, Michael Karin 2025. Protein extraction, co-immunoprecipitation, and immunoblots. **protocols.io** https://dx.doi.org/10.17504/protocols.io.eq2lyx3mpgx9/v1Version created by **Laura Antonucci**

Manuscript citation:
Antonucci, L., Li, N., Duran, A. *et al.* Self-amplifying NRF2–EZH2 epigenetic loop converts KRAS-initiated progenitors to invasive pancreatic cancer. *Nat Cancer* **6**, 1263–1282 (2025). https://doi.org/10.1038/s43018-025-01003-3

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

Created: April 03, 2025

Last Modified: April 07, 2025

Protocol Integer ID: 126124

Keywords: mouse pancreatic tissue, protein extraction, pancreatic tissue, immunoblotting, human cell line, protein

Abstract

Protein extraction, co-immunoprecipitation, and immunoblotting were performed in both mouse and human cell lines, as well as in mouse pancreatic tissue. The main steps used in these experiments are described in the manuscript by Antonucci et al., *NATCANCER-A11229D*.

1 The pancreas or tumor tissues were homogenized in RIPA buffer with protease and phosphatase inhibitors using a Dounce homogenizer (Thomas Scientific, NJ). Cell lines were lysed directly in the same buffer.

After centrifugation, supernatants were collected and separated by SDS-PAGE(1).

Cytoplasmic and nuclear extraction was performed as described(2).

Briefly, pancreata were homogenized in Buffer A (10 mM HEPES pH 7.4, 10 mM KCL, 10 mM NaCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF) supplemented with 0.6% NP40. After centrifugation, the cytoplasmic fractions were collected and the pellets resuspended in 500 µL Buffer B (20 mM HEPES pH 7.4, Glycerol 20%, KCl 100 mM, EDTA 1 mM, DTT 1 mM, PMSF 0.5 mM, leupeptin 10 ug/mL) supplemented with 1% NP40 and the samples centrifuged. The pellets were resuspended in Buffer C (20 mM HEPES pH 7.4, Glycerol 20%, 400 mM NaCl, EDTA 1 mM, EGTA 1 mM, PMSF 0.5 mM, DTT 1 mM, leupeptin 10 mg/mL) to isolate the nuclear fraction. Analysis of EZH2-associated proteins was performed as described(3).

UN-KC6141 cells were lysed in RIPA buffer, and 2mg of protein lysate were immunoprecipitated with 2.5mg of anti EZH2 antibody (Abgent, AM1836A) overnight.

The lysates were then incubated for 2 hours with 20µl of protein G Dynabeads (Invitrogen #10003D).

In the serial co-immunoprecipitations (co-IP), 2mg of protein lysate was first immunoprecipitated with anti EED antibody (E4L6E, Cell Signaling, #85322) overnight, followed by incubation for 2 hours with 20µl of protein A Dynabeads (Invitrogen #10008D).

Eluted proteins were analyzed by SDS-PAGE using the antibodies listed below: rabbit anti-EZH2 (D2C9, Cell Signaling #5246; WB: 1:1000), rabbit anti-ERK1/2 (p44/42 MAPK (Erk1/2), Cell Signaling, #9102; WB 1:2000), rabbit anti-ERK1/2 [Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) Cell Signaling (D13.14.4E) #4370; WB 1:1000, IHC: 1:100], mouse anti-tubulin (Sigma, T9026; WB 1:1000), rabbit anti-histone H3 (ABclonal, A-2348; WB: 1:2000), rabbit anti- 3meH3K27 (ABclonal, A-2363; WB 1:2000), rabbit anti-NQO1 (D6H3A, Cell Signaling #62262; WB 1:1000, IHC: 1:100), rabbit anti-NRF2 (ABclonal, A11159; WB: 1:1000), mouse anti-HSP70 (3A3, Santa Cruz Biotechnology, sc-32239; WB: 1:1000), rabbit anti-EED (E4L6E, Cell Signaling, #85322), rabbit anti-AcH3K27 (D5E4, Cell Signaling, #8173, WB 1:1000), rabbit anti-NOX1 (GeneTex, GTX103888; WB: 1:1000), rabbit anti-4-Hydroxynonenal (4HNE protein adducts, abcam ab46545; WB 1:1000), mouse anti-HSP90 (BD Transduction Laboratories, 610419; WB 1:1000), rabbit anti-

STAT3 (D3Z2G, Cell Signaling #12640; WB: 1:1000), rabbit anti-NF-κB p65 (D14E12, Cell Signaling, # 8242), rabbit anti-E2F-1 (Cell Signaling, #3742), rabbit anti-PPAR gamma (Abcam, ab59256; WB: 1:1000), mouse anti-POL II (CTD448, Santa Cruz Biotechnology, sc-47701; WB: 1:1000), anti-Myc tag antibody (9E10, Abcam, ab32; WB 1:1000), mouse anti-actin (AC-40, Sigma, A4700; WB:1:1000), rabbit anti-aSMA (Abcam, ab5694; WB 1:1000), rabbit anti-RAS (G12D Mutant) (HL10, GeneTex, GTX635362, WB 1:1000, IHC: 1:100).

Protocol references

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