Tandem Affinity Immunopurification

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Abstract

This method is modified from that described by Nakatani Y, Ogryzko V. Immunoaffinity purification of mammalian protein complexes. Methods Enzymol. 2003;370:430-44. Epub 2004/01/10. doi: 10.1016/S0076-6879(03)70037-8 S0076687903700378 [pii]. PubMed PMID: 14712665. It can be used to immunopurify proteins from nuclear extract through 2 sequential rounds of immunoprecipitation. Non-specific interactions are largely eliminated. This protocol is described for Flag, then HA immunoaffinity, but can be adapted for use with other epitopes.

Citation: Xavier Contreras,Marina Srbic,Monsef Benkirane,Rosemary Kiernan Tandem Affinity Immunopurification. **protocols.io**
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Guidelines

This protocol is designed for use with Dignam nuclear extracts prepared from 10 plates (15cm) of HeLa S3 cells stably expressing protein-of-interest tagged Flag and HA but can be adopted for other buffers and tags

Before start

All buffers and samples should be kept on ice or +4°C, all centrifugation steps should be performed at +4°C

Materials

EZviewTM red ANTI-Flag M2 Affinity gel F2426 by Sigma

HA probe (F-7) Sc-7392 by Santa Cruz Biotechnology

Flag peptide 4 mg/ml F3290-4MG by Sigma

HA peptide anti-HA 5 mg/ml 11666975001 by Roche

Cellulose Acetate filter columns Prod #69702 by Thermo Scientific

Protocol

Prepare HA-IP and Glycine buffers

Step 1.

HA-IP buffer

150 mM KCl

20 mM Tris pH 7.5

0.05% NP-40

0.1% Tween

10% glycerol

5 mM MgCl2

prepare, filter sterilize and keep +4°C

add the following just prior to use:

1 mM DTT

CompleteTM (50x stock)

PhosSTOP (10x stock)

Glycine buffer

0.1M Glycine pH 2.2

1M Tris pH 7.9

filter sterilize and keep +4°C

Prepare Flag beads

Step 2.

Take 100 µl of Flag beads per IP

Add ¼ bead volume of 0.1 M Glycine pH 2.2

Mix well (be careful when pipetting so the beads do not stay on the walls of pipette tips)

Incubate for 3 min at RT

Add ½ bead volume of Tris 1 M pH 7.9

Mix well

Pellet beads, centrifuge for 3 min at 3000 rpm at 4°C

Discard supernatant

Wash beads with 1ml of HA-IP buffer

Pellet beads, centrifuge for 3 min at 3000 rpm at 4°C

Resuspend beads in HA-IP buffer

Prepare IP samples

Step 3.

Remove 10% of nuclear extract and store for “input”

Prepare Flag IP sample as follows:

Mix 1 volume of nuclear extract with 2 volumes of HA-IP buffer and prepared Flag beads from step 2.

Flag Immunoprecipitation (IP)

Step 4.

Rotate tubes on rotating wheel for 4 h at 4°C

Pellet beads, centrifuge for 3 min at 3000 rpm at 4°C

Transfer supernatant to prepared cold tubes – “Flag flowthrough” – snap freeze in liquid N_2 and store at -20°C for short term storage or at -80°C for long term storage

Wash Flag beads 5 times as follows:

Add 1 ml of HA-IP buffer to the beads (rotate tube to mix solution)

Pellet beads, centrifuge 3 min at 3000 rpm at 4°C

Discard supernatant

(For last wash, spin tube briefly until 10k rpm so the beads are well pelleted at the bottom of the tube, so it is easier to discard all supernatant)

- this step provides removal of all the proteins that haven’t bound to beads during Flag IP and prevents non-specific binding to beads

Competitive elution with FLAG peptide

Step 5.

Prepare Flag elution buffer (100 µl per elution): dilute Flag peptide to 200 ng/µl final concetration in HA-IP buffer

Add at least 100 µl of Flag elution buffer to Flag beads in 1.5 ml Eppendorf tubes and incubate for 1 h at 4°C on rotating wheel

Pellet beads for 3 min at 3000 rpm at 4°C

Transfer supernatant to prepared cold tube – “E1 Flag elution” (keep at +4°C until you finish with second elution)

Hint: use WB gel loading tips and squeeze the end of the tip with clean sterile tweezers so it is easier to take supernatant without beads

- Adding an excess of Flag peptide to beads allows competitive binding and efficient elution of Flag-tagged protein from the beads

Repeat elution as follows:

Add at least 50 µl of Flag elution buffer to Flag beads in 500 µl tubes and incubate for 1 h at 4°C on rotating wheel

Pellet beads for 3 min at 3000 rpm at 4°C

Remove supernatant “E2 Flag elution” and pool with “E1 Flag elution”

- at this step you should have only Flag tagged protein in your elution

HA Immunoprecipitation (IP)

Step 6.

Prepare HA beads as follows:

(This can be performed during the incubation of the second Flag elution)

Take 40 µl of HA beads and prepare them as described for Flag beads

For each IP sample, resuspend beads in 400 µl of HA-IP buffer

Hint: if you had difficulty removing all Flag beads at the last Flag elution step, add 1-2 µl of Flag peptide so the Flag beads don’t interfere with HA-IP

Mix Flag elutions with prepared HA beads and rotate tubes on rotating wheel for 2 h at 4°C

Pellet beads, 3 min at 3000 rpm at +4°C

Transfer supernatant in prepared cold tube – “HA flowthrough”

Wash HA beads 5 times as follows:

Add 1 ml of HA-IP buffer to the beads (rotate tube to mix solution)

Centrifuge 3 min at 3000 rpm 4°C to pellet beads

Discard supernatant

- this step provides removal of all the proteins that aren't Flag.HA-tagged and prevents non-specific binding of protein to beads

Competitive elution with HA peptide

Step 7.

Prepare HA elution buffer (100 µl per elution): 400 ng/µl final concetration of HA in HA-IP buffer

Add 100 µl of HA elution buffer to HA beads in 1.5 ml Eppendorf tubes and incubate for 1 h at 4°C on rotating wheel

Pellet beads for 3 min at 3000 rpm at 4°C

Transfer supernatant to a cold tube – "E1 HA elution" (keep at +4°C until you finish with second elution)

Repeat elution (second elution) as follows:

Add 50 µl of HA elution buffer to HA beads in 500 µl tubes and incubate for 1h at 4°C on rotating wheel

Pellet beads for 3 min at 3000 rpm at 4°C

Remove supernatant "E2 HA elution" and pool together with "E1 HA elution"

Clear HA elution from beads using Pierce column as follows:

Rinse Pierce column with 400 µl of HA-IP buffer

Centrifuge at 7000 rpm for 3min at 4°C

Discard flowthrough

Load pooled HA elutions onto column

Centrifuge at 7000 rpm for 3min at 4°C

Collect flowthrough and voila, you have your Flag.HA-tagged purified protein

Hint: if you want to check each step of purification by WB you can take a small volume (5%) from each step (Flag flowthrough, Flag E1, Flag E2, Flag beads, HA flowthrough, HA E1, HA E2, HA beads)

of purification and blot against HA and Flag antibodies.