BioTechniques Rapid Dispatches

Protocols

Protein affinity capture from cryogenically disrupted human cells

Protocol for:

Improved methodology for the affinity isolation of human protein complexes expressed at near endogenous levels

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Legend

⇨ *ATTENTION*
* *HINT*
REST

Procedure

Cell extract preparation

Optimized extraction conditions need to be determined on a case-by-case basis for each protein complex under investigation. When doing an affinity capture for the first time we generally start with a 'standard' extraction buffer consisting of: 20mM HEPES pH 7.4, 100mM NaCl, 0.5% v/v Triton X-100. After analyzing the results, further variations on conditions are often explored. All extraction buffers are also supplemented with protease inhibitor cocktails as a general rule.

⇨ *ATTENTION When handling cell grindate, remember to use utensils and tubes pre-cooled with N_2. Tubes containing the cell grindate should always be held on N_2 when not at -80°C.*

1. Weigh out 100 mg of cell powder into a 1.5 or 2 ml microfuge tube.
a. Tare analytical balance with the empty microfuge tube
b. Dispense grindate to tube using an N_2 cooled spoon or spatula
c. Check the mass of the grindate dispensed within tube on the analytical balance

* *HINT* To ease the weighing out of cell powders, small volumetric measuring spoons may be used. These have been found to give reproducible results (see equipment). In any case, weights of grindate dispensed to tubes are quickly checked on an analytical balance, as above.

⇨ *ATTENTION We have found best results using screw-cap microfuge tubes. Residual pressure due to evaporating N_2, held within the cell grindate, can cause standard microfuge tubes to pop open during subsequent warming just prior to the addition of extraction buffer – potentially resulting in loss of the sample.*

2. Open the tube (or loosen screw-cap) with grindate and let stand at RT for 1 min. This will release pressure within the tube and prevent the immediate freezing of the extraction buffer when added to the grindate. No thawing is observed during this 1 min incubation.
3. Add 500 µl of extraction buffer and vortex briefly, and then hold on ice while proceeding to step 4.
4. Use a microtip sonicator to further disperse the grindate (~2 sec, output: 20 W).

⇨ *ATTENTION While vortexing dispenses the grindate into the extraction buffer, depending on the buffer character some cell material aggregation may be observed – presumably membranous material. We have found that dispersing these aggregates provides for the best yield during subsequent affinity capture. A very brief microtip sonication easily disperses these aggregates.*

5. Clarify the extract by centrifugation (e.g. 20,000 RCF, 10 min, 4°C, using Eppendorf 5417R centrifuge).
6. Remove the supernatant (clarified extract) and proceed to the affinity capture. Clarified extract may be briefly held on ice prior to affinity capture, as needed, but in this case it should be transferred away from the pellet to a fresh microfuge tube during the pause.

Affinity capture

Dynabeads are manipulated using neodymium magnets in a specialized microfuge tube holder (see equipment). When placed within the holder, beads are collected at the side of the tube under influence from the magnetic field. Solutions may then be removed without disturbing the beads.

7. Prior to adding the Dynabeads to the clarified extract, they should be pre-washed. Pre-wash 10 µl of Dynabeads slurry, three times w/ 1 ml of extraction buffer. Resuspend the beads by brief vortexing during each wash.

* *HINT Dynabeads can be pre-washed while cell extracts are being centrifuged (step 5).*

8. Transfer the clarified cell extract to tube with beads and vortex briefly.
9. Incubate for 1 h at 4°C with constant mixing (rotator).
10. Aspirate the supernatant and wash the beads three times w/ 1 ml of extraction buffer – as previously, removing the final wash.

* ***HINT*** *After mixing/vortexing, it is helpful to briefly pulse spin the tubes in a mini microcentrifuge to collect all contents to the bottom of the tube (beads will remains essentially suspended), prior to placement on the magnet and aspiration of the solution. Doing so ensures the minimum carryover of solutions.*

11. Elution can be achieved in a number of ways – for direct analysis by SDS-PAGE and Coomassie staining, we favor the following: add 36 µl of 1x LDS and incubate for 10 min, collect and save the supernatant.

* ***HINT*** *Elution from anti-GFP beads requires heating to 75°C during this incubation period, as even 2% w/v LDS (i.e. 1x LDS sample buffer) will not effectively elute GFP-tagged protein from beads conjugated with llama polyclonal anti-GFP Ig. For anti-FLAG Dynabeads, incubation at RT in the presence of 1x loading buffer is sufficient for elution of 3xFLAG-tagged proteins. Heating has not been found to be detrimental, but less IgG leaches from the beads during LDS elution when elution is carried out at RT – hence, RT elution is preferred when possible.*

* ***HINT*** *The volume prescribed has been optimized for eluting from 10 µl of Dynabeads slurry with little loss due to residual liquid trapped within the beads (the beads trap some residue between them in proportion to their volume), and for loading on a 1 mm, 10-well gel. Different amounts of slurry, or different destination gel well sizes may benefit from different elution volumes, optimize as needed.*

⇨***ATTENTION*** *In any case, no reducing agent should be included during elution or excessive amounts of Ig chains will be co-eluted. When using the anti-GFP nanobody the reducing agent should not be a concern, nevertheless, we routinely omit it during elution.*

REST Samples may be frozen at -20°C for brief storage (a few days) or -80°C for extended storage until analysis is desired.

12. Add 4 µl of 500mM DTT, and incubate at 75°C for 10 min, to fully reduce the sample. Load on a gel of your choice.

* ***HINT*** In addition to NuPAGE gels, this loading buffer works well with homemade Bis-Tris and Tris-glycine gels. To provide sufficient space to load all ~40ul of the sample elution, a standard 10-well gel of at least 1mm thickness is required.

Reagents

Antibody conjugated Dynabeads
500mM dithiothreitol (DTT)
Extraction buffer
Liquid nitrogen (N_2)
4x lithium dodecyl sulfate gel loading buffer (Invitrogen, cat. #NP0007)

Equipment

1.5 – 2 ml microfuge tubes (preferably screw-cap)
Microtip sonicator
Bench-top refrigerated microcentrifuge
Sample rotator
Vortex mixer
Neodymium magnet – e.g. Invitrogen DynaMag™-2 magnet, cat. #123-21D (http://www.invitrogen.com/site/us/en/home.html)

Optional

Volumetric spoons – our favorite: Norpro 3080 Mini Measuring Spoons, 5 Piece Set available though www.amazon.com

We have found that one heaping scoop using 'smidgen' gives ~50 mg and one level scoop with 'dash' gives ~250 mg – an initial feel for the 'size' of the scoop must be made by the user.

Received 12 December 2011; accepted 4 April 2012.

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