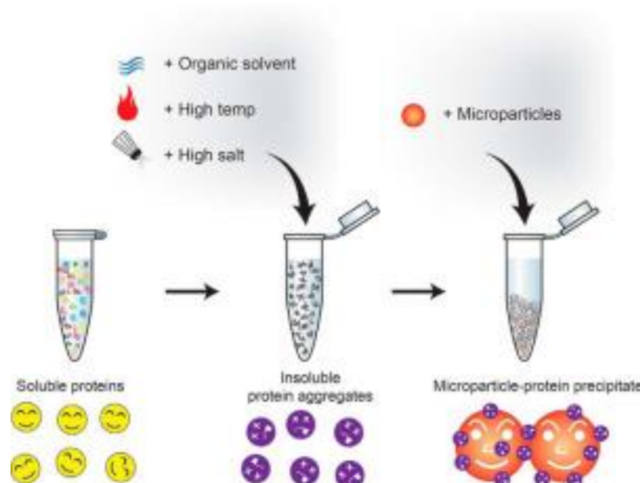
Laboratory Protocol: Protein Aggregation Capture (PAC)

Adapted from Batth *et al*., 2019, https://www.mcponline.org/article/S1535-9476(20)31610-8/fulltext by author Tanveer Singh Batth

University of Copenhagen, Novo Nordisk Foundation Center for Protein Research

This note is not and should not be construed as an endorsement of any product.

This protocol is for on-bead protein aggregation capture in 1.5ml Eppendorf Protein LoBind® tubes, or 96 well plate format, and suitable for use with any standard magnetic rack. Protein extraction can be performed with SDS in biological buffers such as Tris or PBS, with typical quantities ranging from 1% SDS for cells, to 4% SDS for tissues. To maximize recovery, sonication of the sample and/or enzyme-based nucleic acid degradation (using benzonase) is required. It is also recommended that reduction of cysteine residues and alkylation (i.e. with TCEP and CAA) be performed as per standard protocols prior to PAC.



This protocol is optimized for lysates **without** guanidine hydrochloride or urea as extraction agents. Although these are compatible, the sample will have to be diluted to <3M of these agents.

Due the improved handling, rapid separation, and improved retention on magnetic stands, this protocol currently uses ferromagnetic beads, specifically MagReSyn® Hydroxyl beads from ReSyn Biosciences. The use of ferromagnetic beads also ensures this protocol can be translated for automated sample preparation.

1) A protein to bead ratio of 1:4 (weight:weight) is recommended for PAC i.e. for 20µg protein use 100µg (5µl) microparticles. Transfer desired MagReSyn® Hydroxyl microparticles (20mg.ml^{-1} suspension) to 1.5ml Eppendorf Lo Bind tube or 96-well plate.
    - *NOTES*: the protein to bead ratio can be adjusted if necessary, in particular for protocol compatibility for concentrations below 0.1 µg.µl^{-1} you will require approximately 3 times more beads (i.e. protein:bead ratio of 1:15) to improve recovery.
2) Place the tube or plate on the magnetic separator and allow the microparticles to clear (~5-10sec)
3) Remove the storage solution (20% ethanol) and discard
4) Equilibrate microparticles by adding 100µl of 70% ACN and mix by gentle agitation (sufficient to keep the particles remain in suspension).
5) Place the tube on the magnetic separator and allow the microparticles to clear. (suitable for manual, vortex, thermomixer or intellimixer).
6) Remove the wash buffer and discard.
7) Repeat step 4 and 5 for a total of two equilibrations
8) Add protein extract (buffered to a pH of >8) to the equilibrated microparticles
9) Add acetonitrile to the tube containing microparticles and biological extract, to a final concentration of 70% and mix once by pipette to create a uniform suspension.
    - Samples in large volumes (e.g. >500µl) can either be split into smaller volumes in 1.5ml Eppendorf tubes or the aggregation can be performed in a larger vessel such as a 15 or 50ml centrifuge tube.
    - *NOTES*: we have observed that protein aggregation using acetonitrile in a larger tube can lead to protein aggregates accumulating on the tube walls. The aggregation on tube walls is not inherently a problem if this will be covered with the digestion solution.
10) On-bead precipitation should occur within 10 minutes without any agitation.
11) Place tubes/plate on a magnetic rack and allow magnetic separation to occur for ~5-10 sec

(All the subsequent washes should be performed without removing the tubes/plate from the magnetic rack as this result in loss of material).

12) Remove the supernatant by aspiration with a pipette and discard.
    - *NOTES*: make sure not to disturb the separated bead-protein aggregates. Supernatant can be kept for SDS-PAGE analysis in order to check for any unbound proteins (checking efficiency of capture)
13) Add 1 ml of 100% acetonitrile to the tubes and incubate for 10 sec
    - *NOTES*: It is important during the washes that the wash buffers be added gently to the tubes without disturbing the bead-protein aggregate/pellet. Avoid pipetting the wash buffer directly onto the magnetically separated bead-protein aggregate. Number of washes can be increased to 3 for improved purity.
14) Add 1 ml of 70% ethanol to the tubes and incubate for 10 sec
    - *NOTES*: 70-90% methanol or isopropanol can also be used as wash solvents to remove potential contaminants from your protein sample. This step can also be repeated if necessary, to improve purity. Ensure that the wash solvent is completely removed prior adding digest buffer so that it does not interfere with trypsin digest
15) Remove tubes/plate from the magnetic rack and add digestion buffer (50mM HEPES, TEAB, Ammonium Bicarbonate or Tris, pH 8.5) to the tubes and mix thoroughly to form a uniform suspension.
    - *NOTES*: Ensure that any residual beads along the tube walls are submerged in the digestion buffer. Dried beads which adhere to tube walls can be manually displaced by tip until submerged in the digestion buffer.
16) (Optional) Add Lys-C at a ratio of 1:200 (protease to protein) and incubate with intermittent mixing for 1-2 hours at 37°C.
17) Add Trypsin at a ratio of 1:50 (protease to protein) and incubate with intermittent mixing overnight (12-16 hours) at 37°C.
    - *NOTES*: the ratio can be adjusted for increased or decreased as necessary. The digest time can also be reduced to 1-3hrs by using lower LysC and Trypsin ratio such as 1:20 and digesting at higher temperature e.g. 50°C
18) Quench the digestion and acidify the solution to a final TFA concentration of 1%.
    - *NOTES*: This can be achieved by the addition of a 10% TFA stock solution in order to reduce handling of pure TFA solution.
19) Mix and place samples in tubes/plate on magnetic rack for 60 seconds and transfer supernatant to new tubes.
20) Wash beads with 50-100µl 1% TFA for 2min with continuous mixing
21) Pool eluates from step 18 with the digest from step 17.
22) Centrifuge tubes for 10 minutes at maximum microcentrifuge speed (max speed usually 16,000 – 20,000 x G) for 10 minutes.
23) Transfer supernatant to new tubes and clear peptide supernatant using hydrophobic (e.g. C18) or hydrophilic (e.g. HILIC) SPE
    - *NOTE*S: Where a pre-concentration LCMS set-up is available, desalting can be performed via an on-line C18 trap column
24) Residual organic solvent of peptides eluted from hydrophobic SPE can be evaporated and peptides concentrated using a speedvac. Residual DNA or RNA can precipitate at this point, particularly from samples of tissue and certain cell types not treated with sonication and/or enzymatic DNA/RNA degradation
    - *OPTIONAL*: Should you see precipitate, centrifuge resuspended sample at 16,000 – 20,000 x G for 10 minutes.
25) Transfer supernatant to new tubes and analyze by LC/MS/MS

This protocol has been automated on KingFisher® magnetic bead handling systems, and bdz files are available on request.

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