Protein lysate preparation for Mass Spectrometry using BioID2 **Version 1.0 05.10.2023**

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Cell lysate preparation

This process should only begin once a planned date for sample drop off with the ZMBH has been determined.

Stable cell lines or transiently transduced cells can be used; optimization steps necessary for this vary and should be considered on a case-by-case basis.

1. Seed $4x10^6$ cells into a 10 cm dish and culture overnight to allow adherence. For mass-spectrometry, preparing 3 triplicates of each cell line / transfection is necessary.

NOTE: A positive control in the form of cells expressing BioID2 alone should be used each time samples are submitted for mass spectrometry. Plan experiments so that they can be submitted in batches to reduce costs and the amount of reagent necessary for experiments.

2. Treat cells with Biotin (prepared by dissolving in Optimem as a 20x concentrated stock; 12.2 mg Biotin (Sigma# B4501-100MG) in 50 ml Optimem, filter with a 0.22 µm filter) for 16 hours.
3. Wash cells 2x with PBS to remove media and residual biotin.
4. Lyse cells using 500-700 µl M-PER lysis buffer (Thermo Fisher# 78501) with protease inhibitors (Sigma# 11873580001) for 5 mins RT, transfer to protein low bind microcentrifuge tube.
5. Spin 20 min @ 4°C 16000 x g.
6. Transfer supernatant (soluble fraction; which may contain a hazy layer) to a new protein low bind microcentrifuge tube.
7. Store both pellet and soluble fraction at -20°C.
    a. Prepare aliquots of these for testing by Western Blot. Both the pellet and an aliquot of the soluble fraction can be resuspended and boiled in SDS lysis buffers used for Western blot. In practice, only an aliquot of the supernatant is necessary after initial optimization is necessary.

Biotinylated protein immunoprecipitation

Steps at this point are highly variable among different protocols. The steps used here are specifically optimized for the Streptavidin-Sepharose resin beads in this protocol; but largely taken from Roux et al., 2018 (DOI: 10.1002/cpps.51).

Day 1:

1. Thaw samples prepared for immunoprecipitation.

2. Resuspend 100 µl streptavidin-sepharose high performance beads (Cytiva# 90100484) in 1 ml of M-PER lysis buffer with protease inhibitors in a protein low bind microcentrifuge tube to remove ethanol and equilibrate the beads.
3. Centrifuge 2 min at 1000 x g.
4. Carefully remove the supernatant.
5. Add samples to the equilibrated and ensure that the beads are thoroughly resuspended and the pellet at the bottom of the tube is no longer visible by inverting 10-20 times.
6. Incubate beads with cell lysate overnight with end-over-end mixing (using a rocker to mix overnight results in inadequate mixing and will result in beads settling).

Day 2:

1. Prepare an 8M Urea solution (24.024g Urea dissolved into 50 ml buffer of 50 mM Tris-HCl pH 7.4). This solution should be prepared fresh each time.
2. Centrifuge RT 5 min @ 1000 x g to pellet beads.
3. Remove supernatant and transfer to a new microcentrifuge tube.
   Note: It is critical to retain these sample as it will be important in quality checking your samples prior to submission to the core facility.
4. Add 1 ml 8M Urea solution to samples and ensure the beads are resuspended. Incubate 8 min RT with end-over-end mixing.
5. Centrifuge RT 2 min @ 1000 x g to pellet beads.
6. Remove and discard supernatant.
7. Repeat this wash process 3 additional times.
8. Resuspend in 1 ml 8M Urea solution and take a 100 µl aliquot; transfer the aliquot to a new protein low bind tube. The remaining 900 µl can be stored on ice and delivered to the ZMBH mass spectrometry core.
9. The 100 µl should be washed with 1 ml of 50 mM Tris-HCL pH 7.4 for 8 minutes with end-over-end mixing.
10. Centrifuge RT 2 min @ 1000 x g to pellet beads.
11. Remove and discard supernatant.
12. Resuspend beads in 40 µl of SDS lysis buffer.

Quality checking of samples by Western blot

This step is critical to determine sample quality and should be completed immediately.

At least 2 stains are necessary to confirm immunoprecipitation on the streptavidin sepharose; A biotin specific stain as outlined in Roux et al., 2018 and a ligand/tag specific stain. Ensure that these Western blots are submitted on Agilent with the submitted samples.