GUIDELINES AND INFORMATION FOR CUSTOMERS OF CFMP ZMBH

Title: Identification of protein interaction partners using proximity labeling (BioID)

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1. Short description

Proximity labeling (BioID) is employed to investigate protein-protein interactions within complex biological systems. In BioID experiments, a protein of interest is fused with a biotin ligase enzyme, typically the BirA variant. When this engineered protein is expressed in cells, it biotinylates nearby proteins through proximity labeling. Biotinylated proteins can then be isolated and identified using streptavidin-based affinity purification methods, allowing researchers to uncover the spatial and temporal dynamics of protein associations.

Biotinylated, isolated proteins might be separated on the SDS-PAGE and visualized using Coomassie staining. Once the proteins are visualized, the gel bands containing the proteins of interest are excised and subjected to enzymatic digestion, typically with trypsin. The resulting peptides are then analyzed using mass spectrometry. The in-gel approach allows to decrease the amount of co-eluting streptavidin (Protocol A). Streptavidin, which is usually present in excess, may hamper the LC-MS readout. However, internally performed experiments indicate that on-bead digest may still grant higher sensitivity and quantification accuracy (Protocol B).

2. During initial meeting inform us about

- Do you already have iLab account?
- Did you already perform a test experiment? Did you see proteins on the SDS-PAGE gel when stained with Coomassie? Do you know if biotinylation worked?
- What is your control sample?

3. Sample preparation

Below, we provide protocol suitable for efficient elution of the proteins from the beads. Elution buffer is based on the power of detergent (SDS) to disturb molecular interactions. Protocol A allows processing of the samples, which might be contaminated by high excess of streptavidin. In this case, eluted proteins are separated on the SDS-PAGE and streptavidin migrates close to the running front.

Protocol B grants higher sensitivity and quantification accuracy and is uses on-bead digestion. The disadvantage is the presence of streptavidin peptides in the mixture.

Protocol A: In-gel digest

The most efficient way of releasing the proteins from the beads is to use SDS sample buffer. We recommend using **1x SDS sample buffer**. You can use commercial sample buffer or homemade. Here is the recipe for **4x SDS sample buffer**.

Materials:

4x SDS sample buffer:

Component	Volume
Tris (1M, pH 6.8)	10 mL
SDS	4 g
B-mercaptoethanol	10 mL
Glycerol	20 mL
Bromophenol blue	0.1 g
dH_2O	Fill up to 50 mL

Elution procedure:

Mix beads with up to 40 µl of 1x SDS sample buffer. Incubate 10 minutes at 95 °C with shaking. Centrifuge briefly to collect the beads at the bottom of the tube. Transfer the supernatant to the fresh tube.

Note: while working with **membrane proteins**, we recommend to warm sample up to 70 °C or test elution only in presence of SDS (without heating). Membrane proteins tend to aggregate while heated.

Recommendations for running the gel:

- You can obtain an aliquot of fixing solution and colloidal Coomassie if you would like to run the gel in your lab but **you can also run the gel in our facility**. We offer commercial gels (40€ / gel). One can load protein marker and up to **9 samples**. The maximum sample volume is **40 µl.**
- In order to run the gel in our facility, please contact **Sabine Merker by e-mail** and set an appointment**.**
- Always, bring with you **your elution buffer. We strongly recommend** to load **neighboring lanes** with your **elution buffer.**
- To ensure proper separation on the gel, mix your **marker protein** with appropriate amount of **SDS sample buffer.**
- Use **commercial gels** (higher reproducibility and resolution, reduced contamination)

- Run your gel for **1-1.5 cm** (measure the distance from the bottom of the well to the running front). Then simply stop the electrophoresis, rinse the gel shortly with dH_2O, incubate with fixation solution for 20 min with moderate shaking and stain with colloidal Coomassie for 1-4h. De-stain the gel with water by incubating overnight.
- Make a picture of the gel, **mark the lanes on the picture** (preferentially in PowerPoint) and upload it to iLab. File upload is possible after the project request is created.

Protocol B: On-bead digest

Bait protein is bound via biotin/streptavidin
Protein interaction partners are covalently bound
Protocol is optimized for 100µl beads

Materials:

TEAB solution
100 mM TEAB, pH 8.5
Urea solution (10ml)
6 M Urea (MW 60.06 → 3.6 g/10 ml) in 100 mM TEAB, pH 8.5
TCEP solution (1 ml):
100 mM TCEP in Urea solution (MW 286.65 → 28.7 mg/ml)
CAA solution (1 ml):
400 mM CAA in Urea solution (MW 93.51 → 37.4 mg/ml)
Urea Reduction/Alkylation solution (1 ml):
100 µl TCEP solution
100 µl CAA solution
800 µl Urea solution
Lys-C stock solution
200 ng/µl solution (in 0.01% TFA), store at -20°C
Trypsin stock solution
200 ng/µl solution (in 0.01% TFA), store at -20°C
20% TFA stock solution

On-bead digest procedure:

1. Wash as stringent as possible with **1 mL** Urea solution several times (at least 10 times); **detergents have to be completely removed.**
   Notes:
   a. If you use magnetic beads, to properly wash the beads you have to remove the tube from the magnetic rack, mix solution with beads and only afterwards place it again on the magnetic rack.
   b. Do not use autoclaved tubes! Use high-quality tubes from a fresh bug. Sterilization often releases plasticizers, which affect LC-MS analysis.
2. Remove supernatant
3. Add 50 µl Urea-Reduction/Alkylation solution;
   a. Incubate 30min. at room temperature
4. Remove supernatant
5. Wash with 100 µl Urea solution
6. Remove supernatant
7. Add 25 µl Urea solution
8. **Add 300 ng Lys-C è 1.5 µl from stock, 4 hours incubation**
   a. in Thermoshaker, 2000 rpm, 37°C
9. Add 75 µl TEAB solution
10. **Add 300 ng Trypsin è 1.5 µl from stock, from stock, digest over night**

    a. **in Thermoshaker, 2000 rpm, 37°C**
11. Centrifuge at 2000 rpm for 2 minutes
12. Take the supernatant
13. Acidify by adding TFA to 0.4% (vol/vol.) final concentration,
    a. verify that pH is <2
14. Deliver the peptides to the core facility member. Peptides can be stored frozen at -20 °C.

4. General information

- We will provide you the results within 4 weeks from the sample submission
- For BioID experiments, we recommend using at least 3 replicates per condition (e.g. 3 x bait and 3x control). To achieve higher data quality, we recommend using 4-5 replicates. It facilitates the data analysis and interpretation of the results.
- Your samples will be analyzed using a 60 min peptide separation method.