Laboratory 8:
Affinity purification

Objective

- To quantify protein concentration of total cell lysates
- To purify His-tagged recombinant protein

Preparation of cell lysates

24 hours after transfection we collected the culture medium since α-amylase contains a signal peptide and thus will be secreted into the medium. In addition, the cells were harvested and lysed in 400 µl lysis buffer (50 mM Tris pH7.5, 150 mM NaCl, 1% Triton X-100, protease inhibitors) to release cellular proteins.

1. Quantification of proteins using the BCA protein assay

BCA Protein Assay is based on **bicinchoninic acid** (BCA) for the colorimetric detection and quantification of total protein. This method combines the reduction of Cu^{+2} to Cu^{+1} by protein in an alkaline medium with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu^{+1}). A purple-colored reaction product is formed by the chelation of two molecules of BCA with one cuprous ion. This water-soluble complex exhibits a strong absorbance at 562 nm that is linear with increasing protein concentrations over a broad working range (20-2000 µg/mL). To determine the total protein concentration in the cell lysates you will prepare a protein standard curve with known concentrations of bovine serum albumin (BSA).

Materials

- BCA Working Reagent (WR); See BCA Protein Assay Instruction manual on Moodle
- 96-well plate
- BSA stock solution = 2 mg/mL
- Cell lysates from DNA-transfected cells (pAmy2-His and pGFP): **L+**
- Cell lysates from Mock-transfected cells (without plasmid DNA): **L-**
- Lysis buffer (+ protease inhibitors)
- Sterile water
- 1.5 ml microfuge tubes

Procedure

- 1. Prepare BSA standard (dilution series) according to the table below and calculate the missing concentrations :

Tube	Volume of Water (µL)	Volume and Source of BSA (µL)	Final BSA Concentration (µg/mL)
A	0	50 of stock	2000 (= Std.7)
B	25	75 of stock	1500 (= Std.6)
C	50	50 of stock	1000 (= Std.5)
D	50	50 of tube **B**	(= Std.4)
E	50	50 of tube **C**	(= Std.3)
F	50	50 of tube **E**	(= Std.2)
G	50	50 of tube **F**	(= Std.1)
H	50	0	0 = Blank

- 2. Make two dilutions (1:2, 1:5) in separate microfuge tubes of each of the cell lysates (L+ DNA- or L- Mock-transfected) in a final volume of 40 µl lysis buffer, as indicated in the table below.

Amounts in µl	1:2	1:5	1:2	1:5
L+			-	-
L-	-	-		
Lysis buffer				
Total	40	40	40	40

- 3. Label the plate with your group number. Pipette **10 µl** of each standard and each diluted unknown sample (L+ DNA- or L- Mock-transfected, from above) in **triplicates** into a 96 well plate as indicated in the table below.

	1	2	3	4	5	6
A	**tube A**	**tube A**	**tube A**	L+	L+	L+
B	**tube B**	**tube B**	**tube B**	L+ 1:2	L+ 1:2	L+ 1:2
C	**tube C**	**tube C**	**tube C**	L+ 1:5	L+ 1:5	L+ 1:5
D	**tube D**	**tube D**	**tube D**	L-	L-	L-
E	**tube E**	**tube E**	**tube E**	L- 1:2	L- 1:2	L- 1:2
F	**tube F**	**tube F**	**tube F**	L- 1:5	L- 1:5	L- 1:5
G	**tube G**	**tube G**	**tube G**	lysis buffer	lysis buffer	lysis buffer
H	**tube H**	**tube H**	**tube H**			

- 4. Add **200 µl of BCA Working Reagent (WR)** to each well and mix the plate thoroughly on a plate shaker for **30 seconds** (avoid bubbles! Do not mix by pipetting up and down).
- 5. Cover the plate and incubate at **37°C for 30 minutes**.
- 6. Let the plate cool to RT. Measure the absorbance of standards and unknown samples with a spectrophotometer for plates, set at **562 nm** (results will be on Moodle).
- 7. First subtract the A562 value of the blank (water). Next, calculate the mean of the triplicates and create a **standard curve** by plotting the 562 nm values protein standard (y-axis) versus their concentration in µg/ml (x-axis). Aberrant values may be discarded (outliers). Add a trendline within the linear range of the mean values and display equation. Show also raw data points on the same graph.
- 8. Use the standard curve **equation** to determine the **unknown concentration of the cell lysates**. First, subtract the A562 value of the appropriate blank (lysis buffer) and calculate the mean of the triplicates. Aberrant values may be discarded (outliers). In case measurements are outside of the linear range, use the diluted sample to determine the concentration. In this case remember to multiply the concentration by the **dilution factor** to obtain the initial concentration.

Paste the labelled plate reader output (table) and the corresponding standard curve (graph) into your lab notebook. Indicate the protein concentration (multiplied by dilution factor) for both DNA (L+) and mock transfected (L-) cell lysates. For measurements that are outside of the linear range, write ND (= not determined).

Lysate	undiluted µg/ml	1:2 diluted µg/ml	1:5 diluted µg/ml
L+			
L-			

Choose **one dilution** to calculate the volume needed to yield 20 µg of total protein for subsequent analysis by SDS-PAGE in the next lab session. Ideally the triplicates have low variability and the results obtained from different dilutions are consistent.

Lysate	20 µg total protein
L+	_____ µl
L-	_____ µl

Questions

Q1 Are your results from diluted and undiluted lysates consistent? If not, explain.

Q2 Do you expect to get similar yields of total protein for each of your samples (DNA- or Mock- transfected)? Justify your answer.

Q3 Why do you use different blanks in the BCA assay?

Q4 List at least three error sources that may affect the outcome of the experiment.

2. Affinity purification of His-tagged recombinant protein

Purification of α-amylase from cell culture medium on nickel spin columns is based on the affinity of the Ni-NTA resin for recombinant proteins carrying the affinity tag consisting of six consecutive histidine residues (6X His). The recombinant protein is immobilized on the NTA resin by nickel ions that bind to the 6X His tag. Proteins without the tag may bind with low affinity (non-specific binding). Wash steps remove proteins that are bound non-specifically using low concentration of imidazole. For elution, high concentration of imidazole releases tagged proteins from the Ni-NTA resin by competition.

Materials

- Culture medium (700 µl) from pAmy2-His/pGFP transfected cells (collected 24 hours post-transfection) supplemented with 50mM Na_2HPO_4, 150 mM NaCl, 10 mM imidazole **(TM+**)
- NI-NTA spin kit components (Qiagen) : NI-NTA spin column, 2 ml collection tubes, 1.5 ml microfuge tube
- Column equilibration buffer (**CEB**): culture medium supplemented with 50mM Na_2HPO_4, 150 mM NaCl, 10 mM imidazole
- Wash buffer (**WB**; 50 mM Na_2HPO_4, 300 mM NaCl, 20 mM imidazole)
- Elution buffer (**EB**; 50 mM Na_2HPO_4, 300 mM NaCl, 500 mM imidazole)

Procedure

- 1. Equilibrate the affinity column: add **600 µl column equilibration buffer (CEB)** to the NI-NTA spin columns placed in a 2 ml collection tube. Centrifuge for **2 min at 890 x g** (approx. 2900 rpm). Place the NI-NTA spin column into a new 2ml collection tube.
- 2. Transfer **100 µl of culture medium (TM+)** to a 1.5 ml tube, label the tube as indicated below and store at 4°C.
- 3. Load **600 µl culture medium (TM+)** onto the pre-equilibrated NI-NTA column. Centrifuge for **5 min at 270 x g** (approx. 1600 rpm), and collect the flow-through in a 2 ml tube. Transfer the **flow-through (FT)** to a 1.5ml tube, label the tube as indicated below and store at 4°C.
- 4. **Wash** the NI-NTA spin column **twice with 600 µl wash buffer (WB)**. Centrifuge for **2 min at 890 x g** (approx. 2900 rpm), and collect the washes in a 2 ml collection tube. Transfer the washes to a 1.5ml tube, label the tube as indicated below and store at 4°C.
- 5. **Elute** the protein **twice with 300 µl elution buffer (EB)** in a 1.5 ml microfuge tube. Centrifuge for 2 min at 890 x g (approx. 2900 rpm). Label the tube as indicated below and store at 4°C. Column fractions will be analysed in the following lab sessions.

Label your samples as follows with your group number and give to assistant:

- Total medium: Group# TM+
- Flow through: Group# FT
- Wash 1: Group# W1
- Wash 2: Group# W2
- Elution 1: Group# E1
- Elution 2: Group# E2

Questions

1. What is the amino acid sequence of the signal peptide? Use the information on Uniprot, go to PTM/ Processing. In Benchling annotate in ‘features’ the signal sequence in your recombinant plasmid file (name: signal peptide). Provide a screenshot in SLIMS.

2. Look up (online, book etc.) how protein secretion works and the function of the signal peptide during secretion. What is the molecular weight (kDA) of cytoplasmic recombinant AMY2-His? What is the molecular weight of secreted recombinant AMY2-His? In ‘features’ create a translation of the secreted recombinant AMY2-His. To determine the molecular weight generate both proteins in ‘create/ New AA sequence/ forward’ in Benchling. Provide a screenshot in SLIMS.

3. Visit the Human Protein Atlas, an open access database that aims to map all the human proteins in cells, tissues, and organs using an integration of various omics technologies. https://www.proteinatlas.org/ENSG00000243480-AMY2A Based on the 'summary' and 'subcellular localization' for human AMY2A, hypothesize whether mouse recombinant AMY2-His protein is found in the total cell lysate, in the cell culture medium or both? Justify your answer. You will find out in the next lab sessions.