Laboratory 10:
Western blot II / Enzyme assay

Objectives

- To detect a protein of interest using specific antibodies.
- To perform enzyme assays to see whether AMY2 is active.
- To check if the affinity purification of His-tagged recombinant protein was successful.

1. Western blot / Immunoblot

Western blot, also called immunoblot, is an analytical technique used to detect specific proteins. The three main steps in western blotting are 1) separate proteins by size, 2) transfer on solid support, 3) visualise proteins using labelled antibodies.

Antigens are substances that are foreign to an organism and provoke immune responses such as proteins, peptides, some polysaccharides, or also organic substances (pesticides, pharmaceuticals etc.).

Antibodies / Immunoglobulins (Ig) bind specifically to the antigen and are glycoproteins consisting of two identical light and heavy polypeptide chains linked together by disulfide bonds.

Antibody production techniques can be broadly divided into in vivo (living organisms) and in vitro (in lab) approaches. The most common techniques to produce antibodies are:

1. **Polyclonal antibody production:** upon injection of highly concentrated antigens into an animal, antibodies are produced by the immune system (similar to some vaccinations). Antibodies are secreted by different B cell lineages that bind to different parts of the antigen and can be purified from serum (blood).
2. **Monoclonal antibody production (hybridoma technology):** B cells are isolated from the spleen of antigen-treated animals and immortalized by fusion with myeloma cells. The selected clonal hybridoma cell line makes one type of antibody that targets a specific part of the antigen and releases it into the cell culture medium.
3. **Recombinant antibody production:** for antibodies with known amino acid sequence, the heavy and light chains are cloned into expression vectors and expressed in host cells (e.g., HEK293), the monoclonal antibody is secreted in the cell culture medium. This technique is highly customizable and allows to further engineer the variable or constant region.

To visualize the **primary antibody** bound to the protein of interest several methods exist (enzymatic, fluorescent or radioactive). In the present case, we will use a **secondary antibody** conjugated with horseradish peroxidase (HRP). When the enzyme acts on its substrate light is emitted and chemiluminescence can be measured. Secondary antibodies selectively bind to the specific immunoglobulins of a particular species. For example a secondary goat-anti-mouse IgG can be used to detect any primary mouse IgG. Today numerous engineered therapeutic antibodies are available to treat for example cancer, autoimmunity or chronic inflammatory diseases (Zinn 2023).

In order to test whether recombinant α-amylase is produced by transfected HEK293T cells, we lysed the cells, collected the culture medium (remember that α-amylase is secreted), affinity purified His- and Myc- tagged AMY2 and separated the proteins by SDS-PAGE. All proteins extracted from the cells or present in the culture media are on

the nitrocellulose membrane. After their transfer onto a nitrocellulose membrane we use different antibodies to specifically detect AMY2 the tags of the recombinant protein and actin. We use a monoclonal antibody anti-Myc antibody to determine the expression levels of both intracellular and secreted recombinant AMY2. To ensure that samples in cell lysates are equally loaded and transferred, we use anti-actin antibodies as 'loading control', a highly abundant protein that is expected not to differ between the samples. Common other loading controls are tubulin and GAPDH. The relative detection of this "loading control" protein (here actin) is then compared with the signal for the protein of interest (recombinant AMY2). In this experiment we did not include a loading control for the medium.

Materials

- Blocking buffer: 5% powder non-fat milk in PBS + 0.1% Tween20.
- Washing buffer: PBS + 0.1% Tween 20 (PBST)
- Storage buffer: PBS
- Primary antibody solution diluted in blocking buffer:
  1:1000 (v/v) goat anti-actin polyclonal primary antibody
  Different groups will test different dilutions recombinant anti-myc tag antibody or anti-6xHis tag antibody
- Conjugated secondary antibody solutions diluted in blocking buffer: 1:5000 (v/v)
  donkey anti-goat-HRP
  donkey anti-rabbit-HRP
- Orbital shaker
- Substrate for enhanced chemiluminescence (ECL) : Luminol Enhancer and Peroxide Solution
- GeneGnome BioImaging system

Procedure

We have started the procedure. This includes the incubation of the membrane in 20 ml blocking buffer for one hour at room temperature. During this step the proteins contained in the blocking solution will bind to the membrane and prevent non-specific binding of the antibody. Then the blocking buffer was replaced by 10 ml of the primary anti-actin antibody solution and incubated overnight at 4°C.

Always wear gloves when handling the membrane.

- 1. Remove the antibody solution from the blot and wash for 5 minutes with PBST (washing buffer). Repeat this step 2 times (for a total of 3 washes)
- 2. Remove buffer and incubate the membrane in 10 ml secondary antibody solution (donkey anti-goat-HRP and anti-myc-HRP monoclonal antibody) for 45 min on an orbital shaker.
- 3. Remove secondary antibody solution and wash the membrane for 5 minutes with PBST.
- 4. Wash membrane 3 times for 5 minutes with PBS. Keep the membrane moist in PBS (storage buffer) as you prepare the substrate mixture. Do not allow the membrane to dry during the subsequent steps as this reduces the signal.
- 5. Mix 0.5 ml Enhancer with 0.5 ml Peroxide solution (= substrate mix). For best results, use the prepared substrate working solution immediately after mixing. The solution is stable for up to 1 hour at room temperature.
- 6. Incubate the protein side of the blot with the substrate (the ladder is usually stronger on the protein side). Put in a clear plastic wallet to spread liquid evenly and prevent the membrane to dry out.

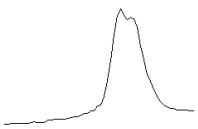
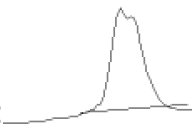
- 7. Incubate aaproximately 5 minutes. The enzyme needs some time to generate the signal
- 11. Visualize the chemiluminescent signal using the GeneGnome imaging system. Take an image of the prestained marker (white light) and the chemiluminescent signal (no light). Create a composite image and export as JPEG.

Image Analysis in FIJI

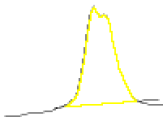
The band intensity in western blots (or other types of gel electrophoresis result) can be used to determine relative protein quantity by comparing bands between different lanes on the same blot. For the purpose of this course you will do the quantification on the composite image- for accurate quantification you should use the raw image of the chmiluminsecent signal.

Open FIJI to quantify the band intensity on the western blot. The commands are found in Analyze > Gels. Start with the actin signal, then repeat the procedure with the signal from recombinant AMY2. Lanes without visible signal cannot be quantified using this method.

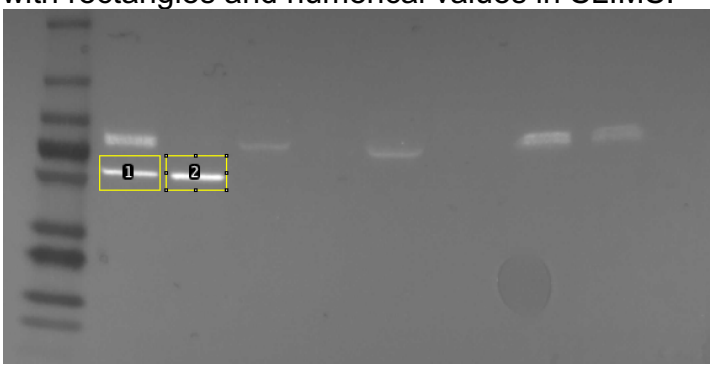
- 1. Open the western blot image in FIJI
- 2. Use the rectangular selection tool  to outline only the actin band in the first lane (here L+). The rectangle height must be at LARGER than half of the width (rectangles with height smaller or equal to one half the width cannot be measured, see step 8). Note the rectangle can only be moved horizontally for the next lanes, make sure that the height covers the signal from all lanes you want to compare (for actin L+ vs L-). For the Myc signal you will compare more lanes, thus the rectangle height must be chosen appropriately.
- 3. Once the rectangle has the correct size and position click Analyze > Gels> **Select First Lane**. The first region of interest will be highlighted with the number 1 (see step 8).
- 4. Click on the first lane and move the rectangle horizontally to the next area of interest (use arrows). Once the rectangle has the correct position click Analyze > Gels> **Select Next Lane**. Numbering of the region will appear. If you have more than two lanes, repeat until all regions of interest are highlighted (for the Myc signal).
- 5. Click Analyze > Gels> Plot Lanes to generate the lane profile plots.
- 6. A new window shows plot profiles for all bands analyzed where peaks correspond to band intensity. Use the line drawing tool  to draw base lines so that each peak defines a closed area. In case the peak was not closed with the line you can ‘Re-plot’ lanes.

peak:  peak closed with line: 

- 7. Measure the areas of the peaks by clicking inside each area (closed peak) in succession with the wand tool  . The measured area is shown with a yellow outline and the peak area shown in the ‘Results’ window as numerical value.

peak area selected with wand tool: 

- 8. For good practice save a copy of the file with the areas measured (save as jpg). Always keep the original file (without lanes highlighted!). Paste image with rectangles and numerical values in SLIMS.



- 9. Copy numerical value from 'Results' window in excel, label data (which image analyzed, band analyzed) and calculate relative band intensity. Set the first lane to 100. For each antibody signal plot a separate bar chart.
- 10. Repeat for the other bands (here: myc-antibody) and calculate relative intensity.

Make a figure with panels A, B, C that contains the original western blot image (no rectangles) annotated, the image with rectangles highlighted and the quantification of anti-actin and anti-Myc signal as bar charts and put into SLIMS.

Questions

Q1 What is the MW of β-actin (check datasheet posted on Moodle) and what do you learn from its presence and quantity in the L+ and L- ? Why is β-actin absent in M + and M-?

Q2 Suppose you incubated your blot with anti-GFP polyclonal antiserum raised in a rabbit. Can you use the secondary antibody donkey-anti-goat-HRP to detect GFP? Justify your answer.

Q3 Based on the western blot, estimate the relative amount of secreted versus intracellular amylase? Take into account the initial volumes of culture medium of transfected cells (2.5 ml / well) and lysis buffer used to prepare cell lysates (400 µl / well), as well as volumes loaded onto the SDS-gel.

Q4 Based on the western blot quantification, which elution fraction contains the most recombinant AMY2 ? Give values.

2. Amylase activity assay

Amylases are glycoside hydrolases which cleave α-1,4-glycosidic bonds. The activity of α-amylase will be assayed by colorimetry using amylose-azure, a non-soluble blue-dyed starch. In the presence of enzyme activity, amylose-azure is hydrolyzed into oligosaccharides (maltose) and solubilized. Absorbance of hydrolyzed substrate can be measured at 595nm using a spectrophotometer. This colorimetric-based assay will allow you to determine if your samples contain active recombinant protein.

Materials

- Amylose-azure; 10 mg/ml in water (Sigma, tube labelled Azure)
- 10X reaction buffer (200 mM KH_2PO_4, pH 7.0, 500mM NaCl, 10mM $CaCl_2$)
- Porcine pancreatic α-amylase (Sigma); 30 ng/µl in 1X reaction buffer (AMY)
- Lysate from GFP+ Amy transfected cells (L+)
- Lysate from Mock transfected cells (L-)
- Medium from GFP+ Amy transfected cells (M+)
- Medium from Mock transfected cells (M-)
- Affinity column fractions:
    - Medium from GFP+His-Amy2 transfected cells (TM+)
    - Flow through affinity column (FT)
    - Wash (W1 + W2)
    - Elution (E1 + E2)
- Controls:
    - Lysis buffer (LB)
    - Culture medium (DMEM)
    - Ni-NTA column equilibration buffer (CEB)
    - Ni-NTA wash buffer (WB)
    - Ni-NTA elution buffer (EB)
- 1.5 ml tubes, 15 ml tube
- 37 °C incubator
- 2 x 96 well plate (flat bottom; Greiner)
- Spectrophotometer (Tecan plate reader measuring A595 nm)

Procedure

- 1. Prepare a master mix for 20 reactions containing amylose-azure, reaction buffer and water into an appropriate tube. Each reaction contains 40 µl amylose-azure and 50 µl sample in a final volume of 200 µl. Fill in the table below.

Note: **Amylose-azure is a suspension and has to be vortexed before each pipetting step!**

Component	Volume (µl) / reaction	Volume (µl) / 20 reactions
amylose-azure	40	
10X reaction buffer	20	
water	90	
Master mix	150	

- 2. Distribute 150 µl master mix into 17 x 1.5 ml tubes.
- 3. Add test samples and controls as indicated in the table below.
- 4. Mix well by vortexing and incubate samples for 30 min at 37°C.
- 5. Mix tubes every 10 minutes (insoluble substrate!).
- 6. Spin down for 1 min at max speed and transfer 100 µl from each supernatant into a 96 well plate. Record how you load the plate. Do NOT transfer insoluble substrate and avoid pipetting samples up and down as it will create bubbles.
- 7. Measure the absorbance at 595 nm. Results will be posted on Moodle.

Amounts in µl **Sample**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Master mix	150	150	150	150	150	150	150	150
H_2O	50	-	-	-	-	-	-	-
α-amylase (Sigma)	-	50	-	-	-	-	-	-
Lysis buffer	-	-	50	-	-	-	-	-
L+	-	-	-	50	-	-	-	-
L-	-	-	-	-	50	-	-	-
DMEM	-	-	-	-	-	50	-	-
M+	-	-	-	-	-	-	50	-
M-	-	-	-	-	-	-	-	50
Final volume	200	200	200	200	200	200	200	200

Amounts in µl **Sample**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**
Master mix	150	150	150	150	150	150	150	150	150
CEB	50	-	-	-	-	-	-	-	-
TM+	-	50	-	-	-	-	-	-	-
FT	-	-	50	-	-	-	-	-	-
Wash buffer	-	-	-	50	-	-	-	-	-
W1	-	-	-	-	50	-	-	-	-
W2	-	-	-	-	-	50	-	-	-
Elution buffer	-	-	-	-	-	-	50	-	-
E1	-	-	-	-	-	-	-	50	-
E2	-	-	-	-	-	-	-	-	50
Final volume	200	200	200	200	200	200	200	200	200

Analysis

- 1. Create a bar chart of enzyme activity for your test samples 1 to 8 and 9 to 17 in Excel. Don't forget to subtract the appropriate background value.
- 2. Create a bar chart of relative activity of intracellular vs secreted α-amylase (L+ vs M+). To allow comparison between samples (of different volumes) don't forget to normalize the background subtracted A595 values to the respective volumes (culture medium of transfected cells (2.5 ml / well) and lysis buffer used to prepare cell lysates (400 µl / well).
- 3. Create a bar chart of relative activity of the affinity column fractions (TM+, FT, W1, W2, E1, E2). Set TM+ to 100%. To allow comparison between samples, normalize the background subtracted A595 values to the respective volumes (affinity column fractions are in 600 µl except elutions that are 300 µl).

Questions

Use the bar charts to answer the questions.

Q2.1 Is the recombinant His-Myc-Tagged AMY2 protein active?

Q2.2 How does the activity compare between lysate (L+) and medium (M+) ? Is the result as expected?

Q2.3 Do you detect enzyme activity in the flow-through (FT). Did the recombinant protein bind efficiently to the affinity column?

Q2.4 Based on the activity assay, which elution fraction contains the most recombinant AMY2 ? Give values. How does the western blot compare to the activity assay? Was the affinity purification successful?

Bibliography

Zinn, S., Vazquez-Lombardi, R., Zimmermann, C. *et al.* Advances in antibody-based therapy in oncology. *Nat Cancer* 4, 165–180 (2023). https://doi.org/10.1038/s43018-023-00516-z