Practice 2: **Recombinant proteins: from small- to large-scale expression technologies**

Location: Loschmidt Laboratories, Kamenice 5/A13, 2nd floor, room 332

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I. WORKFLOW

Selection of an expression host – purpose of protein production – combinatorial screening in small-scale formats – bench-scale and large-scale (fermentor) overproductions – downstream processing and purification strategies – quality/quantity check and final formulation

II. MOTIVATION

Proteins are produced in heterologous systems because of the impossibility to obtain satisfactory yields from natural sources. The production of soluble and functional **recombinant proteins** is among the **main goals in the biotechnological and pharmaceutical industries**. The selection of an optimal expression organism (host) and the most appropriate growth conditions to minimize the formation of insoluble proteins have to be done according to the protein characteristics and downstream requirements. *Escherichia coli* is the most popular recombinant protein expression system despite the great development achieved so far by eukaryotic expression systems. However, it is important to mention that *E. coli* expression system possesses, in many cases, severe limitations for a successful recombinant protein production. Therefore, eukaryotic systems, including mammalian cells, insect cells, yeasts, filamentous fungus, and microalgae, are an important alternative for the production of those difficult-to-express proteins. During this course we will combine theoretical designs with practical demonstrations concerning all aspects of recombinant protein (multi)-expressions, ranging from small- to large-scale (fermentor) overproduction technologies. In the second part, we will focus on the downstream processing and purification strategies. The students will get knowledge on:

- How to select a heterologous system (expression host)
- Pilot small-scale (transient) expression screening to determine optimal expression conditions, and buffer screening strategies
- Scaling-up of production process, bench-scale and large-scale (fermentor) productions
- Harvesting process and timing
- Downstream processing and purification strategies to yield highly pure recombinant proteins in sufficient amounts
- Control (quality/quantity) of the purified protein, final formulation and long-term storage

III. THEORETICAL BACKGROUND

Selection of **expression organism** (**host**): each heterologous expression system has benefits and drawbacks with respect to their capacity for recombinant protein production (**Figure 1**). The gram-negative bacterium ***Escherichia coli*** is frequently the first expression host chosen for the production

of a recombinant protein, owing to the rapid, affordable and technically straightforward culturing associated with its use. The *E. coli* system offers a mean for rapid, high yield, and economical production of recombinant proteins (**Figure 2**). However, high-level production of functional eukaryotic proteins in *E. coli* may not be a routine matter, sometimes it is quite challenging. Techniques to optimize heterologous protein overproduction in *E. coli* have been explored for host strain selection, plasmid copy numbers, promoter selection, mRNA stability, and codon usage, significantly enhancing the yields of the foreign eukaryotic proteins.

Yeasts is a single-celled eukaryotic organism capable of producing very large quantities of recombinant protein. ***Pichia pastoris*** is the most used strain of first choice for yeast expression. The **baculovirus/insect cell expression vector system** (**BEVS**) is a popular choice for the production of recombinant proteins, particularly those requiring complex post-translational modifications or integral membrane proteins. **Mammalian cell-based expression** (e.g. HEK293 cell line) is the dominant system for the production of therapeutic recombinant proteins. Their capacity to handle complex post-translational modifications, folding and assembly of recombinant proteins and protein complexes is superior to other systems. The *Leishmania tarentolae* extract (LTE) *in vitro* translation **cell-free expression** system is a rapid, convenient, flexible and cost effective tool to produce recombinant proteins for biochemical, biophysical and structural analysis.

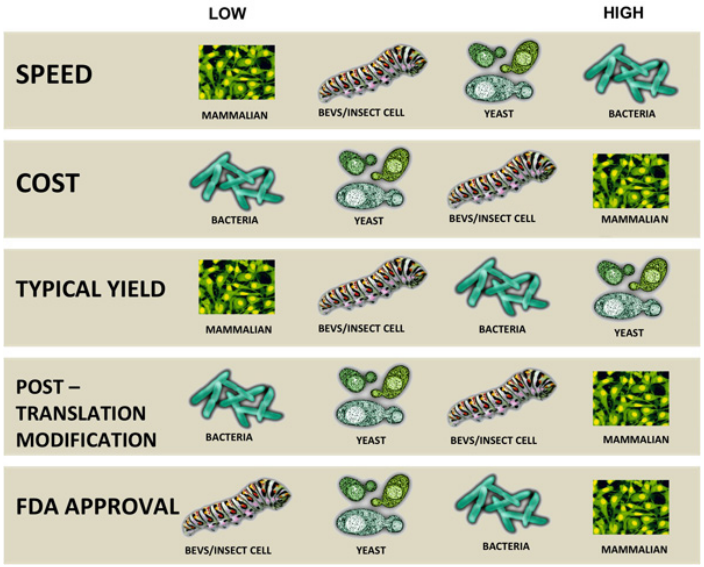

Figure 1. Comparison of the most industrially used expression systems.

At the beginning of a protein expression project, **small-scale expression screenings** in minimal volumes (2-6 mL) are recommended prior to running large-scale overproductions. The pilot small-scale expression tests are designed to test different expression parameters (host strain selection, timing of induction and harvesting, expression temperature, pH, ionic strength etc.) to find an optimal condition and buffer screening. **Large-scale expression** in bacteria, yeast, insect and mammalian cell systems in culture volumes from 400 mL to 10 L are available in shake flask and from 2 L to 20 L in a stirred-tank bioreactor or up to 25 L in a Wave bioreactor. Large-scale production in **bioreactor** (**fermentor**) is very complex procedure where several factors play a crucial role on the performance of the culture. Composition of cultivation broth, sterilization efficiency, proper agitation and aeration, temperature or feeding strategy can all together influence final yield of biomass and recombinant protein from the fermentation. Since culture in fermentor is half opened system, it is very susceptible to contamination. It is very important for the operator of the fermentor to maintain sterility throughout the fermentation. In this practice, the students will learn how to assemble the fermentor vessel and prepare it for sterilization, how to calibrate pH and DO (dissolved oxygen) sensors and set up the fermentation together with creation of recipes for fed-batch cultivations.

Recombinant protein expression in *E. coli*: basic principles and advantages versus limitations

ADVANTAGES

- Inexpensive setup and running costs
- High recombinant protein production levels
- Short timeline from cloning to protein recovery (1 week)
- Limited technical knowledge required for culturing
- Scalability from small (2 mL) to very large industrial culture (>10,000 L) volumes

DISADVANTAGES

- Inability to perform post-translation modifications (PTMs)
- Limited formation of disulphide bond

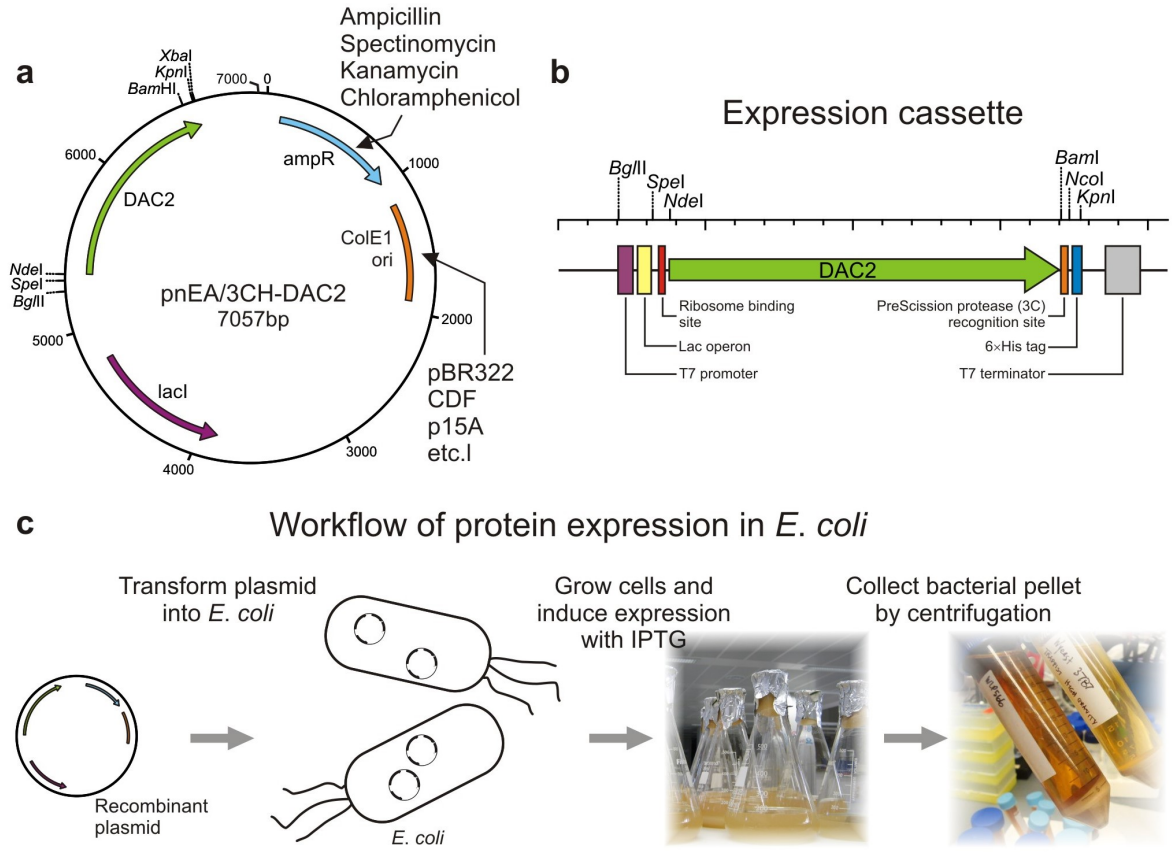

Figure 2. Schematic representation of the plasmid used for the protein expression in *E. coli*. (**A**) Map of the typical expression plasmid. The gene of interest (*DAC2*) is inserted between *Nde*I and *Bam*HI restriction sites. Selection in *E. coli* is performed by the beta-lactamase ampicillin resistance gene (ampR). Origin of replication sequence (ColE1) is available for maintenance in *E. coli* cells, and the lacI gene is present for expression of the Lac repressor protein. (**B**) Details of the expression cassette. The gene of interest (DAC2) is controlled by the T7 promoter and T7 terminator. The *DAC2* gene is in cloned in frame with a sequence coding for a C-terminal thrombin cleavage site followed by a poly-histidine tag. (**C**) Overview of recombinant protein expression workflow in *E. coli*.

Recombinant protein purification: the aim of a purification procedure is to obtain a highly pure and stable protein at an appropriate concentration in a buffer compatible with the intended downstream application (**Figure 3**). Chromatographic techniques are the most powerful and commonly used means of purifying recombinant proteins. Each technique separates proteins based on different properties, so it is often advantageous to combine several types to maximise separation of the

recombinant protein from host cell proteins. The use of **fusion tags** (polyhistidines, Strep-tag, GST, MBP, SUMO, thioredoxin etc.) not only facilitates affinity chromatography steps, but also can dramatically improve protein expression, stability, resistance to proteolytic degradation and solubility. Variety of **proteases** (thrombin, protease 3C, TEV etc.) for fusion tag removal during downstream processing is available.

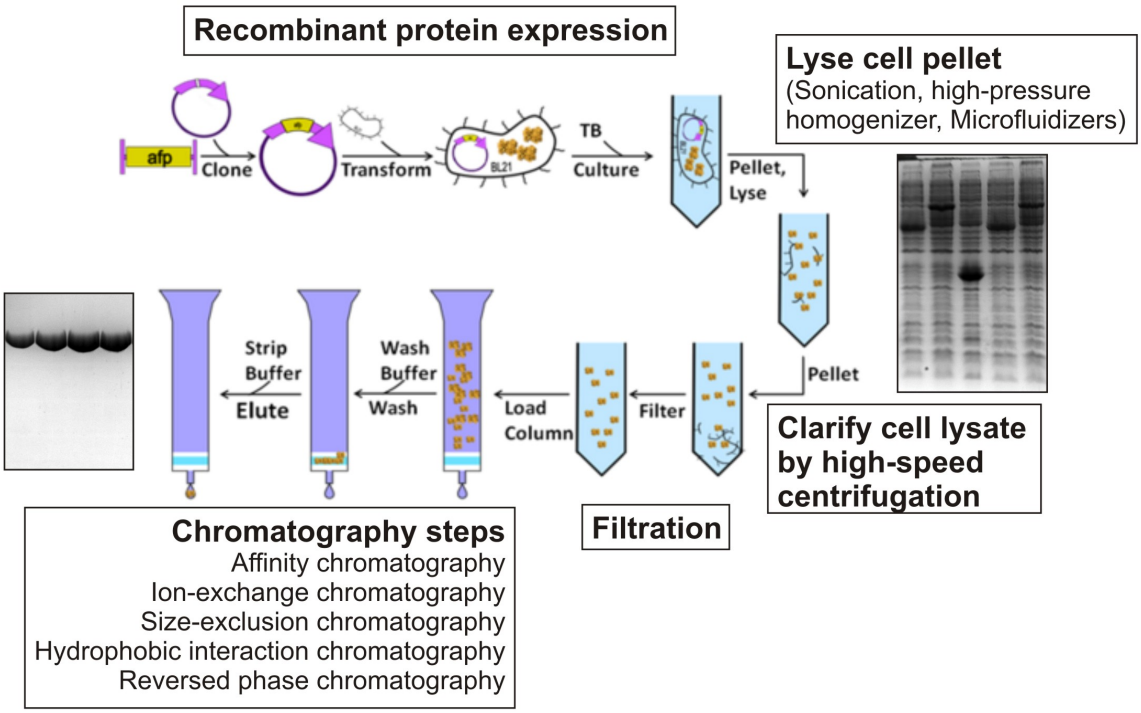

Figure 3. Workflow of recombinant protein purification includes (i) the lysis of a cell pellet by sonication or high-pressure homogenizer, (i) the clarification of cell lysate by high-speed centrifugation, (iii) filtration, and (iv) one or more chromatographic purification steps.

Recombinant protein characterization: an aggregation problem. A key challenge in recombinant protein production is to maintain and store the target protein in a soluble and stable form. Protein aggregation can compromise protein function and thus it is necessary to overcome this challenge to generate functionally active protein (**Figure 4**). Protein aggregates can be detected by (i) analytical size-exclusion chromatography (SEC), (ii) dynamic light scattering (DLS), and (iii) analytical ultracentrifugation (AUC).

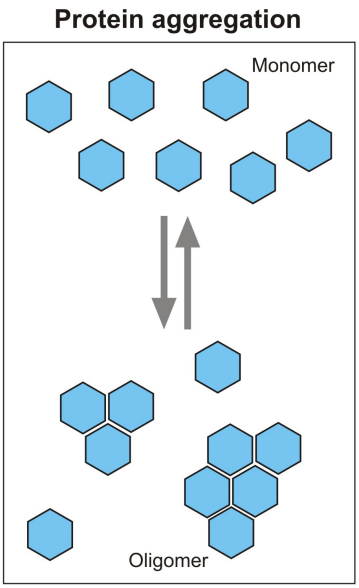

Figure 4. Schematic representation of unwanted protein aggregation problem that can compromise protein (enzyme) function.

How to avoid (minimize) the protein aggregation problem:

- Culture conditions (e.g. reducing temperature)
- Buffer composition (ionic strenght, pH, reducing agents)
- Presence co-factors (Acetyl-CoA, metal ions)
- Fusion tags (Trx, MBP, SUMO)
- Minimising sample handling
- Avoiding time delays between purification steps
- Performing purification steps at 4°C
- Store purified proteins in -80°C

!!! Concluding remark: The recombinant protein production project is ultimately determined by the end-use of the recombinant protein. The overall success of a project lies in an effective project design.

IV. THEORETICAL DESINGS AND PRACTICAL DEMONSTRATIONS

Small-scale expression tests: finding optimal condition and buffer screening

1. Day 1, the *E. coli* BL21 (DE3) cells are transformed with the expression plasmid. Transformed bacteria are seeded on a 6-well plates containing 2xLB agar medium with ampicillin (100 µg/ml); the plates are then incubated at 37°C overnight.
2. Day 2, ampicillin-resistant colonies are inoculated in minicultures (2 ml 2xLB, 0.5% glucose and 100 µg/ml ampicillin).
3. After incubation (275 rpm, 37°C) for 6h, induction is done at 22°C overnight by adding 2 ml of 2xLB medium with 0.6 % lactose, 0.5 mM IPTG, 20 mM HEPES pH 7.4 and ampicillin 100 µg/ml.
4. Day 3, cells are centrifuged (3,500 rpm, 10 min), re-suspended in four different lysis buffers (10 mM Tris-HCl pH 8.0 with 50 mM, 100 mM, 200 mM or 400 mM KCl) and lysed by sonication (1 min, 40 % amplitude).
5. The lysates are clarified by centrifugation (4,000 rpm, 20 min), and then the supernatants are loaded on Talon Superflow Metal Affinity Resin (Clontech) pre-equilibrated in the lysis buffer.
6. The samples are incubated on roller shaker (4°C, 2 h), and the resins are then washed with the corresponding lysis buffers twice by centrifugation (1,000 rpm, 2 min).
7. Finally, the resins are re-suspended in Laemmli Buffer (40 µl), and the proteins bound on the resin are analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and visualised by Coomassie Brilliant Blue staining.

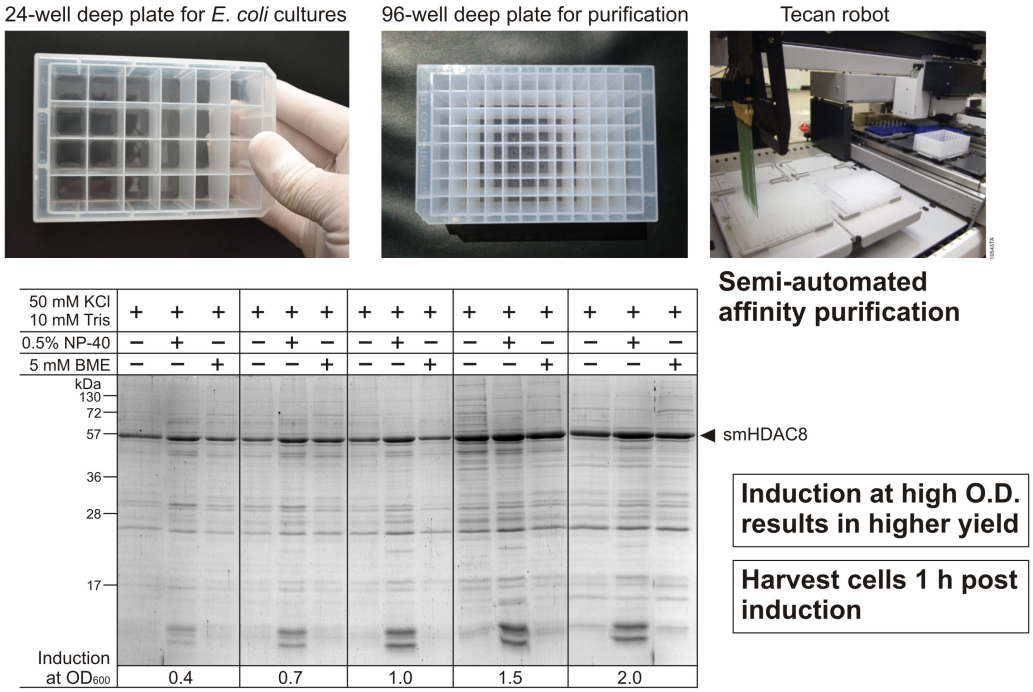

Figure 5. The example of small-scale expression screening in 24-well plates that revealed induction and harvesting times as the key parameters in successful production of soluble smHDAC8 protein.

Protocol for large-scale recombinant protein production in bioreactor (fermentor)

Protocol for assembly of the fermentation vessel:

1. Prepare and dissolve all components of the fermentation broth. The volume of the medium should not be higher than working volume of the fermentor (usually 2/3 of total volume).
2. Calibrate pH probes (See Protocol for calibration of pH probes).
3. Pour dissolved medium to the vessel, add antifoaming reagent Struktol SB2020 to the final concentration 100 µL/L.
4. Insert baffles to the vessel. Make sure the O-ring is present on the top of the vessel.
5. Attach the lid to the glass part of the vessel by tightening large screws on the top of the lid.
6. Attach small plastic tubes to reagent inlet ports and sampling port, and big tubes to the sparger inlet and gas exhaust. Fix by tightening of screw clamps if necessary.
7. Attach the pH and DO probes to the vessel lid.
8. Place rubber septa to remaining ports and fix them with metal rings.
9. Close all plastic tubes with clamps, close sparger inlet tube with Hofman screw clamp.
10. Insert air filter to sparger inlet and gas exhaust tubing, cover ends with aluminium foil. Cover all plastic tube ends with aluminium foil.
11. Make sure all ports and tubes are closed, except for exhaust tube.
12. Sterilize the fermentor in autoclave.
13. After sterilization, let the fermentor cool down to room temperature.

Protocol for calibration of pH probe:

1. Connect the pH probe to the connector on fermentor control unit.
2. Select calibration mode on the control panel.
3. Put the probe in the container with calibration solution with pH 4.1.
4. Wait until the signal is stable, confirm the value.
5. Rinse the probe with distilled water.
6. Repeat measurement with calibration solution with pH 7 or 9.
7. Confirm new calibration slope.

Protocol for preparation of seed cultured:

1. Prepare night culture by picking one colony of freshly transformed *E. coli* BL21(DE3) cells to 10 mL LB medium with respective antibiotics.
2. Cultivate the cells overnight at 37 °C and 200 rpm.
3. Approximately 8-10 hrs prior to fermentation, prepare seed culture by transferring 2 mL of night culture to 200 mL of LB medium with respective antibiotics.
4. Incubate at 37 °C and 200 rpm.

Preparation of the vessel for fermentation protocol:

1. Fill up the jacket with tap water if necessary.
2. Connect the hose connectors to the thermostat.
3. Connect all probes to appropriate connectors on the control unit.
4. Turn on heating by setting the fermentation temperature (30 °C in our case).
5. Attach the motor on the top of the lid.
6. Wait until the DO probe is polarized.
7. Calibrate DO probe (See Protocol for calibration of DO probe).
8. Connect tubes to appropriate pumps and solution bottles (citric acid and NaOH).
9. Connect the sparger inlet tube to mass flow control.
10. Release all clamps on tubing with exception of clamp on sampling tube.
11. Set the cultivation parameters on control panel or in computer.
12. Inoculate fermentor with seed culture. For this purpose, seed culture volume corresponding to 1/100 of volume of medium in fermentor is used.
13. After 1 hr, add 2 g/L lactose to induce expression of heterologous protein. 14. Let the fermentation run overnight, harvest the cells by centrifugation and store the culture at -80 °C.

Protocol for calibration of DO probe:

1. Wait until the temperature of the medium reaches demanded temperature. Be sure the stirring is ON.
2. Connect bottle with pure nitrogen to sparger inlet tube via the sterile filter.
3. On control panel, select DO probe calibration module.
4. Wait until medium is fully saturated by nitrogen. Collect signal for 0 % O_2.
5. Connect air tube to the sparger inlet.
6. Wait until the medium is saturated with air. Collect signal for 100 % O_2.
7. Confirm new calibration slope.
8. This calibration has to be done prior to every fermentation! Oxygen solubility differs with medium composition and viscosity and its temperature.

Settings for batch fermentation:

1. Temperature 30 °C
2. Stirring cascade parameters: 30 % saturation, setpoint 500, minimum 500, maximum 1000 rpm
3. Aeration 0.5 vvm
4. 4. pH 7

Protocol for sampling from the fermentor:

1. Connect the sterile syringe to sampling tube. Release the clamp. Suck ca. 5 mL to rinse the tube with fresh culture. Fix the clamp again. Unplug the syringe and discard whole volume. Repeate this to take your sample. Transfer the culture from syringe to sterile glass tube.
2. Transfer 1 mL of the culture to a plastic cuvette and measure optical density at 600 nm.
3. Prepare 6 tubes with 900 µL of PBS buffer. Transfer 100 µL of the culture to first tube, mix properly. Transfer 100 µL of suspension from first to second tube, repeat until the sixth tube.

Take 100 µL of the final suspension and spread it with glass spreader on two plates with Plate Count Agar. Incubate the plates overnight at 37 °C.

4. Pre-weigh 15 mL plastic falcon, transfer 10 mL of the culture to the falcon and centrifuge for 5 min at 5,000 g. Discard supernatant and weigh the cells.
5. Re-suspend the cells in 1 mL of water, transfer the suspension to pre-weighted aluminium foil. Put the foil with suspension for 2 hrs to dryer (110 °C). Weigh the foil again, calculate the dry cell weight.

Equipment: Biostat B Plus bench-top fermentor (Sartorius Stedim) – Labfors 3 bench-top fermentor (Infors HT) – table centrifuge – ultracentrifuge Avanti J30I (Beckman-Coulter) – LabStak M10 membrane filtration system (AlfaLaval) – spectrophotometer and plastic cuvettes – bench-top dryer STZ 5,4 (FALC)

Purification of His-tagged recombinant protein

1. The following protocol is provided considering the use of cell pellets from 3 L of cultures. First, if required, thaw the re-suspended cell pellets. Adjust the volume of the cell re-suspension to 40 mL per liter of culture (i.e. final volume of 120 mL for 3 L of culture) using the lysis buffer (identical to the resuspension buffer).
2. Lyse the cell suspension using a high-pressure homogenizer at high pressure (18,000 psi) using a single round of lysis. After the lysis, centrifuge the disrupted cell suspension at 210,000 × g for 1 h and collect the supernatant in an ice-cold bottle.
3. Apply the supernatant to a column with 2 ml of Talon Metal affinity resin pre-equilibrated in lysis buffer. Briefly, connect the column with pre-equilibrated Talon resin to a peristaltic pump and pump the supernatant from Step 1 through the column with Talon resin at a flow rate of 4.0-5.0 ml/min. Every 30 min, disconnect the column from the peristaltic pump and mix the resin to release the excess pressure. After the loading, wash the column extensively with approximately 100 ml of the lysis buffer to remove non-specifically bound proteins.
4. Release the protein from the Talon resin by thrombin treatment. Briefly, re-suspend the Talon resin with bound smHDAC8-His fusion protein with the lysis buffer and transfer it to a new sterile 15-mL Falcon tube. The volume of the resin suspension should be approximately 5 ml. Add 60 µl of thrombin (1U/ µl) and place the tube on a rolling mixer overnight at 4°C.
5. Next morning, separate the released protein from the Talon resin particles by applying the resin suspension onto an Econo-Pac column and collect the unbound flow-through fraction into a fresh sterile 15-mL Falcon tube. Wash the resin with additional 3 ml of the lysis buffer to harvest all thrombin-released protein. Check the presence and concentration of protein in the flow-through fraction by the Bio-Rad Protein Assay.
6. Load the flow-through containing smHDAC8 enzyme from the Step 4 onto a 1-mL HiTrap Q FF column pre-equilibrated with low-salt ion-exchange chromatography buffer (10 mM Tris-HCl pH=8.0; 50 mM KCl). Elute the bound protein with a gradient of KCl (50 mM to 1 M KCl): see **Figure 5A** for a typical ion-exchange purification of smHDAC8. Identify fractions containing the protein by SDS-PAGE.
7. Pool the peak fractions from the ion-exchange chromatography from Step 5 and load this sample onto a gel filtration column (16/60 Superdex 200) equilibrated with gel filtration buffer. Identify fractions containing the target protein by SDS-PAGE. See **Figure 5B** for a typical gel filtration purification of smHDAC8.
8. Pool the peak fractions from gel filtration from Step 6, and concentrate the smHDAC8 protein with an Amicon Ultra centrifugal filter unit to reach a final concentration of 2.5 mg/ml. Check purity of the purified smHDAC8 enzyme by SDS-PAGE and determine protein concentration

by the Bio-Rad Protein Assay reagent. Flash-freeze the final product with liquid nitrogen and store at -80°C.

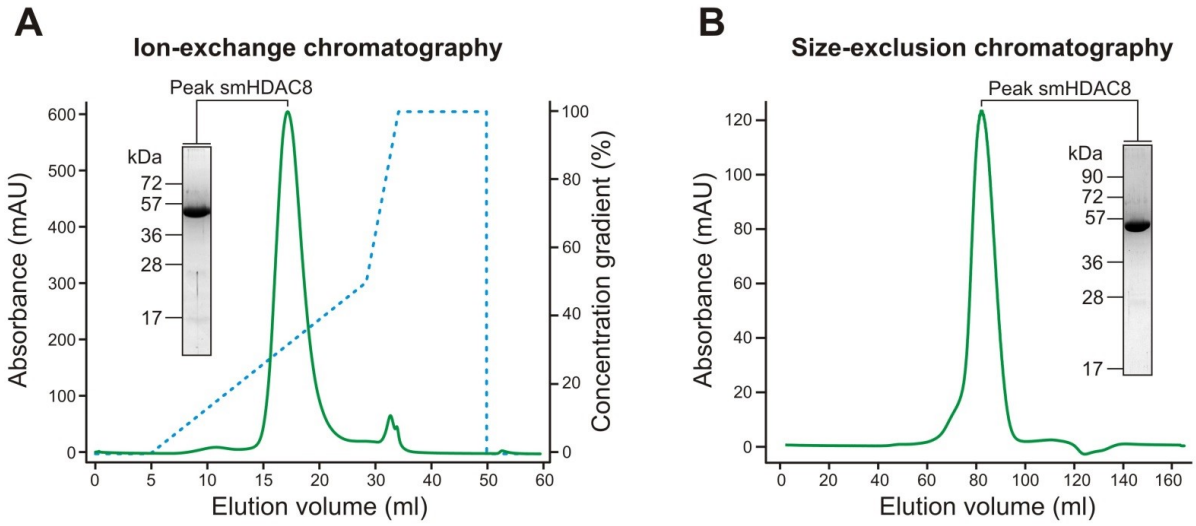

Figure 5. (**A**) Chromatogram of ion-exchange purification of smHDAC8 protein. The gradient used for this purification step is displayed (blue broken line). (**B**) Chromatogram of gel filtration purification of smHDAC8 protein.

Quality/quantity control of the purified protein

1. **Protein concentration** is measured on DeNovix^{R} DS-11 Spectrophotometer (DeNovix Inc., USA) using the absorbance A_{280} mode, where molar extinction coefficient (ε) and molar mass (Mr) are provided.
2. Proper protein folding is detected by **circular dichroism** (**CD**). CD spectra are recorded at protein concentration 0.2 mg.mL^{-1} and at 20 °C using a spectropolarimeter Chirascan (Applied Photophysics). Data are typically collected from 185 to 260 nm, at 100 nm/min, 1 s response time and 1 nm bandwidth using a 0.1 cm quartz cuvette. Each spectrum shown is the average of five individual scans and is corrected for absorbance caused by the buffer. Collected CD data are expressed in terms of the mean residue ellipticity (Θ_{MRE}) using the equation

$$\Theta_{MRE} = \frac{\Theta_{obs} \cdot M_w \cdot 100}{n \cdot c \cdot l}$$

where Θ_{obs} is the observed ellipticity in degrees, M_w is the protein molecular weight, n is number of residues, l is the cell path length, c is the protein concentration (in mg/ml) and the factor 100 originates from the conversion of the molecular weight to mg/mol.

3. **Dynamic light scattering** (**DLS**): The dynamic light scattering (DLS) experiments are conducted typically with protein solutions (1-2mg/ml) in a corresponding buffer containing using instrument DynaPro NanoStar (Wyatt). Protein solutions are centrifuged (13,000 rpm/10 min) prior to DLS measurement in order to remove impurities. Before measurement temperature is equilibrated to 20°C.
4. **Differential scanning fluorimetry** (**nanoDSF**) measurements: Thermal stability of recombinant proteins is analysed by a label-free differential scanning fluorimetry (DSF) approach using a Prometheus NT.48 instrument (NanoTemper Technologies). Briefly, the shift of intrinsic tryptophan fluorescence of proteins upon gradual temperature-triggered

unfolding (temperature gradient 20–95°C) is monitored by detecting the emission fluorescence at 330 and 350 nm. The measurements is carried out in nanoDSF-grade high sensitivity glass capillaries (NanoTemper Technologies) at a heating rate of 1°C/min. Protein melting points (Tm) are inferred from the first derivative of the ratio of tryptophan emission intensities at 330 and 350 nm.

VI. LITERATURE

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V. HOMEWORK

Your goal is to recombinantly produce of 2 g of highly pure haloalkane dehalogenase DhaA enzyme from *Desulfobacterium autotrophicum*. See amino acid and corresponding nucleotide sequences below:

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>ACN15444.1 DhaA [Desulfobacterium autotrophicum HRM2]
MVTRDPAEQSRNIKSPGIRRKINGTMVGTKDFYEIYPFVPHFMTLDRHKLHYLDLGKGSPVVMVHGNPTWSFYFRRLARDLSV
NHRVIVPDHMGCGLSDKPSTRDYDYTLASRVRDLDRLIQSLDLGKKITLVVHDWGGMIGCAWALRHLDRIDRIIITNTSGFHL
PGAKRFPLRLWLIKYLPWFAIPGIQGLNLFARAALYMAPKQSLSTTVRQGLTAPYNSWKNRIATLKFVQDIPLSPRDKSYELV
NWVDTHLEGLKTVPMMILWGRHDFVFDLSFLDEWNKRFPHAQTHIFEDAGHYLFEDKPDETSNLIKKFIEEY

>CP001087.1:2679075-2680040 Desulfobacterium autotrophicum HRM2, complete genome
ATGGTAACCAGGGATCCAGCGGAGCAAAGCAGAAACATCAAAAGTCCGGGCATCAGAAGAAAGATCAACGGCACCATGGTCGG
CACCAAGGATTTTTATGAAATATATCCCTTTGTTCCCCATTTCATGACCCTGGACCGGCACAAACTCCACTACCTTGACCTGG
GTAAGGGAAGTCCAGTTGTCATGGTCCACGGTAATCCCACCTGGTCGTTTTATTTTCGCAGGCTTGCCCGGGATCTTTCGGTG
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ACAAACCCGATGAAACATCAAATCTTATCAAAAAATTCATAGAGGAGTACTAA
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1. Select a suitable expression host (heterologous system) for the DhaA enzyme overproduction and explain why the selected host is the best choice:

2. Propose and design a strategy for the DNA template synthesis, including primer design:

3. Propose a cloning strategy – ligation-dependent versus ligation-independent cloning, selection of expression vector, affinity/solubility tags etc.? How will you check the error-free clones?

4. Briefly describe production process – how will you introduce a foreign gene into the host, from pre-culture to large-scale overproduction, inducible versus stable expression, cytotoxicity issue, timing, harvesting strategy etc.

5. How will you determine the quality and yield of the purified enzyme?

6. How will you determine oligomeric state of the DhaA enzyme?