2. Experimental procedure

OBLIGATORY:
Please watch on YouTube the clip "Automatic Pipet | MIT Digital Lab Techniques Manual" explaining how to use automatic pipets. You can find it under this link: https://youtu.be/AcNtVgOp0hI

Material:

- Competent cells of *E. coli* M15 (pREP4)
- Plasmid pQE32-Eos
- Sterile LB (culture medium for bacteria)
- Sterile agar
- Antibiotic solution (ampicillin 100 mg/ml, kanamycin 50 mg/ml)
- IPTG (1 M)
- Ni^{2+}-charged resin
- Chromatography column
- Buffers for protein purification:
    - Lysis buffer (20 mM Tris-HCl pH 7.5, 300 mM NaCl)
    - Elution buffer (20 mM Tris-HCl pH 7.5, 300 mM NaCl, 300 mM Imidazole)

Wear gloves and a lab coat!

2.1 Transformation of competent *E. coli* M15 (pREP4) cells with plasmid pQE32-Eos

Day 1:

- Thaw two aliquots (one sample, one negative control) of competent *E.coli* M15 (pREP4) cells on ice for 5 min.
- Add 1 µl of the plasmid to the sample cells and mix by pipetting up and down.
    - No plasmid is added to the negative control.
- Incubate both aliquots on ice for 5 min.
- Heat shock both aliquots for 45 sec at 42 °C.
- Incubate both aliquots on ice for 5 min.
- Add 1 ml of sterile LB to each aliquot.
- Incubate for 1 h shaking at 37 °C.
- In the meantime, prepare two agar plates and label them: Sample and negative control.
- Plate 100 µl of the sample and the negative control on one agar plate each.
- Incubate the plates over night at 37 °C.

Preparation of two agar plates:

- Melt 25 ml 2x agar in the microwave and mix with 25 ml 2x LB.
- Cool the mixture down to about 40 °C.
- Add 50 µl of ampicillin and 25 µl of kanamycin.
- Mix by pipetting up and down.
- Pour the medium in 2 different agar plates.

2.2 Bacterial growth and Eos overexpression

Day 2 morning:

5 ml preculture:

- Mix 5 ml sterile LB with 5 µl ampicillin and 2.5 µl kanamycin.
- Inoculate the medium with bacteria grown on the agar plate: Scratch the plate using a sterile pipet tip and drop the tip into the test tube.
- Let the bacteria grow at 37 °C and 200 rpm for 6 h.

Day 2 afternoon:

100 ml culture:

- Prepare 100 ml sterile LB with 100 µl ampicillin and 50 µl kanamycin.
- Inoculate the medium with the bacteria from the preculture (OD_{600}=0.06).
- Cultivate the cells at 37 °C and 200 rpm shaking and measure the OD_{600} every 20 min (show the growth curve in your report).
- When OD_{600} is at 0.5 - 0.7, add 100 µl IPTG to the culture.
- Grow over night at 25 °C and 200 rpm shaking.

Activates the lAc operon & starts Synth of our protein!

checking OD
Charge cuvette w. 200µL of Sol. & 200µL of Ref. in diffent vials

2.3 Cell lysis and protein purification

Day 3 morning:

Harvesting of the cells and cell lysis:

2 x 50 ml of bacteria solution

- Harvest the bacteria culture by centrifugation (10 min, 3000-6000x g, 4 °C)
- Discard the supernatant and put the pellet on ice.
- Add 30 ml lysis buffer to the pellet and resuspend the pellet in the buffer.
- Lyse the cells by sonication on ice:
  - Use the Branson Sonifier® in lab N25/3204: 1 min effective sonication time with pulses of 15 sec at 30 % power (amplitude) with resting on ice for 30 sec between the pulses. → We also cooled it to prevent photobleaching.
- Centrifuge the sample (20 min, 20000x g, 4 °C). The supernatant (cytosol) should contain the protein. → lunch break

many issues, possible insufficient Sonication

Protein purification (Nickel affinity chromatography):

- Wash 0.5 ml mixed Ni^{2+}-charged resin per group once with water and once with lysis buffer (centrifugation for 2 min at 1000x g).
- Add the cytosol to the resin and incubate it in a rotating wheel (10 min, 4 °C).
- Centrifuge (2 min, 1000x g) and carefully remove the supernatant.
- Resuspend the resin in 0.5 ml lysis buffer, transfer it to a spin column by pipetting and then centrifuge (1 min, 1000x g).
- Wash the resin with 0.8 ml lysis buffer and centrifuge (1 min, 1000x g).
- Remove residual wash buffer by centrifuging again (1 min, 1000x g).

done by CAM.

- Transfer the spin column to a fresh tube, add 0.8 ml elution buffer to the resin, incubate for 2 min and then centrifuge (1 min, 1000x g).

2.4 Protein quantification and verification

Day 3 afternoon:

Recording of the absorption spectrum and calculation of the protein concentration:

Prior to the experiment: Look up the extinction coefficient of Eos at 280 nm and 506 nm.

- Measure the absorption spectrum of the protein solution using a UV spectrometer (*Cary Varian 50*) in the range 250 nm-600 nm, $OD_{506} < 1$.
  Use the 'Advanced Settings': Scan rate: medium.
  First, scan the elution buffer as a blank (baseline).
- Calculate the protein concentration using the molecular extinction coefficient of Eos (use Beer-Lambert law).
- Dilute the protein such that the final concentration is 1 µM in 200 µl final volume.

Day 4:

Photoconversion of Eos:

CAUTION: The laser with a power of up to 100 mW can **irrevocably damage** the **human eye! Therefore, it must be operated only after the lid of the sample chamber was closed!**

In order to verify that the product is in fact Eos, an experiment is performed, which shows that the green-emitting protein is converted to a red-emitting protein upon illumination with UV light.

- Fill the above-prepared solution (≈1 µM Eos) into a special black fluorescence cuvette (1 cm path length, 9 mm beam height, open side window).
- Place the cuvette into the photoconversion setup for monitoring fluorescence from both the green and the red form of Eos.
- Start OceanView software and open project 'Fast_Photoconv', which contains the following settings:
  - 'integration time': 50 ms (reduce, if saturation (>60.000) occurs)
  - 'scans to average': 20 (update time is then 1 second)
  - 'boxcar width': 5 (corresponds to ≈1 nm spectral resolution)
- Configure graph saving (file symbol with tool on the right). Most settings are already done in the project 'Fast_Photoconv' but **the bold items have to be set manually!**
  - **Set Target directory: group#** (directory has to be generated before)
  - File format: ASCII (plain)
  - File suffix: file counter
  - Save: every scan
  - Stop after this many scans: 60
  - Apply. Exit.

- Rescale the graph to the range 400-700 nm (magnifier symbol with the '2' inside)
- **Push the rightmost button 'view trendline time trail'**

○ When the cuvette is in place:

Start data acquisition by the **'start all file writers'** symbol and close the lid of the sample chamber, which closes a contact to **switch on the laser** (4.5 V corresponding to ≈33 mW of laser power)

→ 'File saving in progress' will appear at the lower left

(Note: most of the settings can by changed during acquisition, even the stopping conditions!)

Keep the box closed while the laser is running

○ When the measurement is finished:

- Move the series of 60 spectra to a USB stick (2-column text-files)
- Produce at home a graph showing a few spectra (~5-10) at different stages with corresponding times indicated.
- Produce a kinetics graph (intensity over time) for the peak intensity of the green and the red species.

LITERATURE:

[1] Alberts, B. *et al.*: "Molecular Biology of the Cell", Garland Science, 5th edition

[2] Lakowicz, J.R.: "Principles of Fluorescence Spectroscopy", Kluwer Academic/Plenum Publishers

[3] Sambrook, J.: "Molecular Cloning", Cold Spring Harbour Laboratory Press, 2000.

[4] Wiedemann, J. *et al.*: "EosFP, a fluorescent marker protein with UV-inducible green-to-red fluorescence conversion", PNAS, 2004

[5] Nienhaus, G.U. *et al.*: "Photoconvertible Fluorescent Protein EosFP", Biophysical Properties and Cell Biology Applications. Photochemistry and Photobiology, 2006.

1°	0.107	00
2°	0.130	
3°	0.205	
4°	0.[illegible]244	
5°	0.448	
6°	0.675	
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