



POTENTIATING THE ITALIAN CAPACITY FOR STRUCTURAL BIOLOGY SERVICES IN INSTRUCT-ERIC

PROTOCOL TO PRODUCE ALPHA HUMAN CARBONIC ANHYDRASE 2

IO2.1.4 Expression protocols

Activity 2.1: Development of a platform for a high-throughput protein expression

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Call for proposals MUR n. 3264 in date 28/12/2021

Mission 4 – Education and Research Component 2: from research to business

Investment 3.1 Fund for the realisation of an integrated system of research and innovation infrastructures

Action 3.1.1 Creation of new research infrastructures, strengthening of existing ones and their networking for Scientific Excellence under Horizon Europe

RI : INSTRUCT-ERIC – Integrated Structural Biology Infrastructure

ESFRI domain: Health and Food

RI at high priority

Funding Admission DD MUR n. 115 in date 21/06/2022

Starting Date: 1 November 2022

Duration of the project: 30 month

ID Proposal: IR0000009

CUP: B53C22001790006

Protocol to produce alpha Human Carbonic Anhydrase 2

1. Transformation

- 1) Mix 1 μL of pCAM coding the gene of human Carbonic Anhydrase (approximately 100 ng/ μL) with 50 μL of **BL21(DE3)** cell strain in a sterile eppendorf.
- 2) Incubate the culture on ice for 30 minutes.
- 3) Incubate the culture in a 42 °C bath for 30 seconds and back to ice again.
- 4) After 2 minutes on ice, add 1 mL of LB media to the culture.
- 5) Incubate the culture at 37 °C, 180 rpm, for 1 hour.
- 6) Plate 200 μL of the grown culture into a LB-agar plate supplemented with ampicillin and 1 % glucose.
- 7) Incubate the plate at 37 °C overnight.

2. Expression (Marley method) (Ratio 2 LB : 1 M9)

- 1) Pick an isolated colony from the LB-agar plate from the previous day and resuspend in 500 μL of sterile water.

OBS: This can be done also from a glycerol stock; however, the labeling yield will be lower.

- 2) Re-plate 50 μL of the previous culture into two new LB-agar plate supplemented with ampicillin and 1 % glucose and incubate at 37 °C overnight.
- 3) In the next day, scrape both plates and resuspend in 20 mL of sterile LB-media.
- 4) Inoculate 10 mL of the previously prepared culture in to 1 L LB media, supplemented with ampicillin and 1 % glucose.

OBS: OD 600 nm at this point should be around 0.12.

- 5) Incubate at 37 °C 160 rpm.
- 6) When OD reaches 0.6, centrifuge the cultures at 4000 rpm for 15 minutes.
- 7) Discard supernatant and resuspend pellet in 1 L of M9 supplemented with MgSO_4 , CaCl_2 , 3 g Glucose (13C or not), 1.2 g 15N-Amonium sulfate, 0.5 mM ZnSO_4 and ampicillin.
- 8) Incubate at 37 °C 160 rpm for 30 minutes.
- 9) Add 1 mL of 1 M IPTG (1 mM IPTG final concentration).
- 10) Incubate cultures at 25 °C, 160 rpm overnight
- 11) Centrifuge the culture at 7500 rpm for 20 mins.
- 12) Store cells at -20 °C.

3. Extraction

- 1) Resuspend the cell pellet in 70 mL of buffer, 20 mM Tris-SO₄, 500 μ M ZnSO₄ pH 8.
- 2) Sonicate for 30 seconds with a resting period on ice for 3 minutes (repeat for 10 times).
- 3) Ultracentrifuge the lysate at 40000 rpm for 40 minutes.
- 4) Discard the pellet and filter supernadant with a 0.45 micra filter.

4. Purification by Histrap (5 mL)

- 1) Wash the column for 5 CV of water, then 5 CV buffer B, then CV buffer A.
- 2) Load the sample into the column
- 3) Wash the column with 5-10 CV Buffer A
- 4) Perform a gradient from 0 % to 50 % imidazole in 15 CV.
- 5) Wash the column with 5 CV Buffer B
- 6) Collect the pure fractions.
- 7) Concentrate purified sample with a 10 kDa Amicon centricon device until it reaches 10 mL.

5. Purification by Size Exclusion Chromatography 75

- 1) Equilibrate the column with 1.5 CV of Buffer C.
- 2) Load purified sample from previous purification into the column.
- 3) Elute the sample with Buffer C.
- 4) Collect the pure samples
- 5) Store at 4 °C.

6. Demetallation protocol

- 1) Concentrate sample to around 1 mL with 10 kDa Amicon centricon device.
- 2) Add the concentrated protein to a 49 mL solution containing Buffer D.
- 3) Keep solution at 4 °C overnight.
- 4) the next day, buffer exchange the sample into Buffer E, through successful concentration and dilution method using 10 kDa Amicon centricon device.

OBS: Recommended to concentrate down to 0.5-1 mL and then do 10x fold dilution of the sample with buffer E, then repeat process around 10 times or if all organic/inorganic “contaminants” are around the nanomolar concentration.

List of buffers

Buffer A: 20 mM Tris-SO₄, 500 μ M ZnSO₄, pH 8

Buffer B: 20 mM Tris-SO₄, 500 μ M ZnSO₄, 500 mM Imidazole, pH 8

1 liter: 2.42 g Tris, 1 mL ZnSO₄ (0.5 mM Stock), then add sulfuric acid until pH 8.

Buffer C: 50 mM Sodium Phosphate, pH 7

Buffer D: 200 mM Sodium Phosphate, 50 mM Dipicolinic acid, pH 7

Buffer E: 10 mM HEPES, pH 6.8

Other

MW = 29246 Da

$\epsilon_{280\text{ nm}} = 50\,420\text{ M}^{-1}\text{ cm}^{-1}$

OBS: these are the values for the WT-hCAII

Consideration notes:

This expression protocol was only tested for the double mutant hCAII (H3N and H4N) and not for the wild-type and neither triple mutant (H3N, H4N and H64N). However, same results are expected for the expression. Previous expression protocol produced roughly 33 % of labeled protein and this one around 90 %. The problem is the plasmid. It seems that when the culture reaches an unknown density, it starts expression without the inducer. We changed the strain from RIPL to BL21(DE3) and added 1 % of glucose in every step, which acts as a repressor, before the addition of the inducer