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SZABVÁNYMŰVELETI ELŐÍRÁS

Protein expression and purification protocol for recombinant human dihydrolipoamide dehydrogenase

Készítette:

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Dr. Ambrus Attila
tanársegéd

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Dr. Ádám Veronika
intézet igazgató

Dátum

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minőségirányítási megbízott

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MÓDOSÍTÁSOK JEGYZÉKE

Módosította Dátum/Aláírás	Változat száma	Módosított oldalszám	Jóváhagyta Dátum/Aláírás	Kibocsátás időpontja

Nyilvántartott példány: ☐ Munkapéldány: ☐ A példány sorszáma: ☐

TARTALOMJEGYZÉK

1. A MUNKAUTASÍTÁS/PROTOKOLL CÉLJA

Összefoglalja a rutinszerűen alkalmazott fehérjeexpressziós technológiai eljárás részleteit.

2. A MUNKAUTASÍTÁS/PROTOKOLL ÉRVÉNYESSÉGI TERÜLETE

Orvosi Biokémiai Intézet Neurokémia részlege.

3. ILLETÉKESSÉG ÉS FELELŐSSÉG MEGHATÁROZÁSA

A dokumentum kidolgozásáért felelős:
Részlegvezető
A dokumentum alkalmazásáért felelős:
Kutatók, oktatók
A dokumentumban foglaltak végrehajtásáért felelős:
Laborasszisztensek, kutatók, oktatók
A dokumentumban szabályozott tevékenység rendszer felülvizsgálat alkalmával történő felülvizsgálatáért felelős:
A Minőségirányítási vezető

4. FOGALMAK MEGHATÁROZÁSA

Nem szükséges külön fogalmak meghatározása.

5. A MUNKAUTASÍTÁS/PROTOKOLL LEÍRÁSA

Protocol for protein expression and processing of human dihydrolipoamide dehydrogenase and its mutants

Written by: Mattias Nilsson, Tommy Ivanics, Zoltán Klimaj and Attila Ambrus

Note: When handling chloramphenicol and/or bacteria ALWAYS use gloves. Also remember not to touch items unnecessarily with gloves, and those that you have to touch take it as a habit to wipe them off with EtOH when you are finished.

Note2: Always change pipette tip if you have entered fluid that will contaminate your stock or specimen.

Preparation of LB medium

We need:

- 5X2L Erlenmeyer flasks, spoon, weighing boat, aluminum foil, paper cork, autoclave strip
- Millipore water, LB medium (powder)
- Tara-balance

Steps:

- first we have to rinse the flasks two times
- Measure out roughly 14 g of LB into each flask. *Take care of LB powder as it easily gets airborne.*
- add 700 ml Millipore water, *shake gently to solubilize the powder (it will be dissolved completely when autoclaved)*
- close the flasks with "paper cork", cover them with aluminum foil and stick autoclave-strip on them

Autoclaving

We need:

- Containers to be autoclaved

Steps:

- Check water level inside the autoclave and in the bottle on the outside
- Open and close the emergency vent
- Put the containers into the baskets (two in each basket, bend them if necessary); make sure the aluminum foil is not torn
- Close lid to hand-tight level
- Set it:
  - Temperature: 121 degrees
  - Time: 30 min
- Start the machine
- **Note: be careful during and after autoclaving to avoid burns, use thick gloves (provided next to the autoclave); open the lid slowly releasing steam away from you**
- Store autoclaved vessels under the hood

Agar plate preparation

We need:

- Empty, sterile Petri dishes (14 pieces in general)
- Agar
- Chloramphenicol (30 mg/ml dissolved in EtOH, stored at -20 ^{o}C)
- 15 ml centrifuge tube

Steps:

- Prepare LB medium, as explained earlier, now with 150 ml distilled water, 3 g LB and 2.25 g Agar
- Prepare it for autoclaving
- Autoclave it
- Prepare as sterile environment as possible on bench (with help of EtOH) and then put the plates there

- Afterwards let the autoclaved flask cool down until you can hold it without getting burned (app. 45-50 degrees). Then add 150 µl 30 mg/ml Chloramphenicol (1:1000).
- Grab the centrifuge tube in your dominant hand, holding it with 3-5th digits. Then with your thumb and index, of the same hand, remove the paper plug and pour with your non-dominant hand 10 ml into the centrifuge tube and then replug the container.
- Open the plate with non-dominant hand and pour content in plate, *taking care to minimize the time that the plate is open*. *Swirl the plate around so that the fluid covers the entire bottom*.
- Repeat preceding two steps for all 14 plates
- Open the plates a little for avoiding the formation of water-drops, leave to stand for ~20 min, leave room (to minimize air-disturbance)
- Return and close lids, turn the plates so the agar is on the ceiling of the plate and cover the plates individually with parafilm
- Put them on one another, wrap aluminum around the pile and put in +4 ^{o}C fridge

Transformation

We need:

- Ice
- Competent cells from -80 ^{o}C (on ice), vector plasmid from -20 ^{o}C (also on ice)
- EtOH with glass-spreader and matches
- Sigma LB-medium (as liquid found in +4 ^{o}C fridge)

Steps:

- Start the thermoblock on 42 ^{o}C
- Thaw competent cells on ice
- Pipette out the (transformation-ready) 2,5 µl aliquot of DNA (use setting of 5µl) and add it to the competent cell aliquot, homogenize with pipette *by sucking up and down and stirring gently in the Eppendorf tube*
- Incubate on ice for 30 minutes
- Put tube in thermoblock for **exactly** 45 seconds then immediately back on ice for 2 min
- Change the thermoblock temperature to 37 ^{o}C and set shaking to 200 rpm
- Remove tube from ice and add 900 µl LB-medium
- Incubate for 1 hour in thermoblock
- Pipette out 500 µl from tube onto an agar-plate carefully. *Keep exposure of plate to minimum*.
- Remove the glass spreader from EtOH and ignite the EtOH that remained on glass spreader. Put spreader back in EtOH and flame it one more time.
- Use glass spreader to spread the liquid evenly over plate after cooling the spreader down on gel distant from bacteria
- Mark the name of the construct on the lid, then put the plate to 37 ^{o}C for incubation
- After 10 min (more if not most liquid is absorbed in gel) turn the plate upside-down (with the agar on top) to avoid water dripping during incubation
- Leave it there for 2-3 days, check them the next morning and daily afterwards

Overnight culture

We need:

- 30 mg/ml Chloramphenicol solution (from -20 °C fridge)
- agar-plate with colonies
- Sterile non-filter 10 µL pipette tips in red box
- 70 mL sterile LB medium

Steps:

- The shaker is set to 12 °C and 200 rpm
- Prepare 70 µl pipette (1/1000 of medium)
- Get Chloramphenicol from freezer and use a non-sterile centrifugal tube to keep the container with chloramphenicol in it → 70 µl to LB
- Find a good colony on the agar plate. *A good colony is medium sized, isolated and more towards the middle*
- Using a sterile non-filter 10 µL pipette tip, touch the colony that the colony material would go in the tip a bit and/or stuck on it
- Release the tip into the LB medium
- Mark the designation of the transformant (e.g. name of mutant) on the container

Inoculation

We need:

- 5X2L containers with LB media (sterile)
- 30 mg/ml Chloramphenicol solution
- Non-sterile 50 mL centrifuge tube
- Overnight culture

Steps:

- The LB media (2L containers) are put into the 12 °C shaker to cool down to prevent heat shock of the culture
- Put 700 µl chloramphenicol (1/1000) into each vessel. Use non-sterile tube as a tube-holder
- 1/50 (e.g. 14 ml if 700 ml is used) of the overnight culture is transferred to each big vessel
- Mark name of construct on containers

OD-measurement

We need:

- 2 plastic cuvettes
- Pipettes
- Sigma LB-medium from +4 °C fridge
- spectrophotometer

Steps:

- Switch on the UV-VIS two-beam spectrophotometer 15-30 min before measurement
- Switch on the computer
- Log in using the username: TRETTER, no password

- Start the program "Parancsikon UV". When a message comes up: press "kihagyás"
- Choose Method → 550-ATI → Data collection
- Fill each cuvette with 1 ml of LB medium
- Go to spectrophotometer. Take off one glove and open the spectrophotometer with this hand. Then put both cuvettes in the spectrophotometer. Note: take care of the orientation of the cuvette that light would go through the non-shaded sides
- Press F9 on the computer to zero the absorbances
- Take out the cuvette closer to you and remove 500 µl from it
- *Grab the pipette, with your dominant hand, by the top and so that you can press the top. Remove the plug of one of the containers in the shaker with your non-dominant hand. Try to use a wider-mouth vessel to the left front corner of shaker to make sampling easier. Pipette out 500 µl preferably without touching the glass or anything but the media (sterile tip!).* Add this to the cuvette with 500 µl LB. Mix the content of the cuvette by pipetting up and down several times (for homogenized cuvette content).
- Put the cuvette back into the spectrophotometer
- If the absorbance is higher than 0.3 (corresponding to 0.6 in the culture) → **Induction**. If the value has not reached 0.3, repeat the above procedure until it does (duplication of *E.coli* at 12 ^{o}C can vary between 4-8 h)

Induction

We need:

- AHT (anhydrotetracycline) in -20 ^{o}C freezer. Note: light-sensitive

Steps:

- 70 µl (1:10,000) of AHT is put into each of the culture-containing containers

Harvest of bacteria

We need:

- the 5 flasks (induced bacteria culture), that are ready for harvest (generally after 24 h of induction)
- four 500 mL centrifuge tubes
- balance, plastic Pasteur-pipette for balancing tubes before centrifuging
- Beckman centrifuge with JA-10 rotor (pre-chilled)

Steps:

- first we have to rinse the tubes three times
- mark on the tubes what they contain, *e.g. E3-326*
- switch on the centrifuge, set it:
  - rpm: 6,500
  - time:15 min
  - rotor: JA-10, -> 10
  - temperature: 4 ^{o}C, let it cool for ~10 min
- take two flasks that contain 700 ml culture each, effuse them into the four tubes (~350ml, the foam as well), balance the tubes in couples, put the balanced tubes in couples to face one another in the centrifuge

- put the cap of the rotor on, close the roof, check the settings, then start the machine, stay there until speed is stable
- during centrifuging, prepare one of the empty flasks with bleach
- after centrifuging, decant the supernatant into the bleach filled flask, and take care of the pellet (try to cover pellet with another batch of cold culture immediately)
- repeat the previous steps as needed
- after two centrifugations we have one more 700 ml flask, that we need only two tubes for, so we quickly take the other two tubes with the pellet into the fridge at -20 ^{o}C, then centrifuge the remaining two tubes in accordance with the above mentioned steps
- all four tubes are in the fridge that contain the pellet from the five large flasks

Preliminary purification

We need:

- the four 500 mL tubes with pellet from -20 ^{o}C
- potter, spoon, Buffer P, two 50 mL centrifuge tubes, 1.2 μm syringe filters, syringe, ice, pipette

Steps:

- rinse the potter and the centrifuge tubes
- take the 500 mL tubes with the pellet from the fridge and scrape the pellet into the potter, fill the potter with 35 ml cold Buffer P (use another 15 mL to rinse the potter rod after finishing homogenization)
- the steps from here on are carried out on ice, where that is possible, to keep low temperature
- move the potter gently to homogenize the pellet, do not get too much bubbling because it destructs bacteria (we need to eliminate the outer membrane of *E.coli* only by EDTA-containing Buffer P), then incubate on ice for 30 min; at 15 min shake the fluid around a bit.
- during this time, set the centrifuge:
  - rpm: 15,000
  - time:30 min
  - rotor: JA-20, -> 20 (pre-chilled)
  - temperature: 4 ^{o}C, cool everything for ~10 min
- effuse the content of the potter into the two centrifuge tubes, balance the tubes by Pasteur-pipette, put the balanced tubes in couples to face one another into the centrifuge
- start the machine
- get the supernatant, that contains the protein, and filter that through 1.2 μm filters into a 50 mL volumetric cylinder standing in ice (this goes to FPLC)
- in the filtering process, change the filter when it is too hard to push the syringe, at the end of the process grout some air through the filter to get the remaining solution

FPLC

We need:

- wet Kimwipes
- Laptop
- Buffers W1-3, Buffer R, Buffer E in 50 mL centrifuge tubes
- Empty 50 mL centrifuge tubes (wash, flow-thru), empty 15 ml centrifuge tube (elution), a 500 mL empty beaker (waste), all closed by double parafilm

Steps:

- Turn on computer, start Unicorn 5.11
  - System control↓
  - Run
  - Show details
  - Values at main: Strep-Tactin macro prep 5ml column (set alarm to 7.0 MPa)
  - Usually start with Pump/wash A,B
  - Equilibrate with 10 CV (column volume) Buffer W (75-100 ml)
  - System Pressure at 7.0 MPa
  - Sample introduction: 1-2 ml less than actual sample (~45-50 mL)
  - Wash column: with 75 ml
  - Length of elution: 3 CV (15 ml)
  - After-elution volume: 7 ml
  - Regeneration (Buffer R): 45 ml
  - Wash off of regeneration buffer (Buffer W): 45 ml
  - Next->next->next
  - Name: generally "(name of protein)+date"
- Check lines (clear of air-bubbles) then "Start"
- After preliminary wash, change to sample then "Continue"
- Change to collecting in the 50 mL centrifuge tube (flow-thru) from Waste
- After sample administration, change to buffer W1
- Change centrifuge tube from flow-through to wash
- When the wash tube is full, change tubing back to Waste (only the beginning of the washing step contains considerable amounts of protein, anyways)
- Refill washing buffer (Buffer W1) if it gets low and the baseline is still not low enough and flat
- When wash has baselined, change to elution (Buffer E)
- When UV-meter spikes showing eluting protein then change tubing to a 15 mL centrifuge tube (all collection tubes are closed by double parafilm that should always be stabbed through when changing collection tube) and when close again to baseline then pause, switch to Buffer W2 and continue. In case of successful elution, throw away flow-through and washes
- Wait at least 10 ml Buffer W2, then change to Buffer R (45 ml) then switch to Buffer W3 (also 45 mL)
- After this run put piston washing lines in the big container with water while the buffer containing lines back to the bigger Buffer W container ("kiindulási" Buffer W). If not used for a while, store the system in 20 % EtOH/H_2O (pump washes A, B and a fake program run to rinse lines, too). All solutions should be sonicated for 1 h in the sonicator bath and then chilled at 4 ^oC for overnight before use.
- Close program in computer, turn off lights on fridge and turn off the FPLC

- Note: every time the line goes into a new fluid, wipe it clean with a new, wet (ddH_2O) Kimwipe.

Buffer exchange

We need:

- swing-rotor centrifuge
- 4 mL concentrator tube (MWCO=30 kDa)
- Elution from FPLC purification
- 50 mM K-PO_4, pH=7.3 buffer
- 1 mL non-sterile pipette tips and pipette
- ice

Steps:

- Chill down centrifuge to 4 ^oC
- Fill tube with 4 mL elution; fill counter balance tube with 4 mL water
- Spin at 4,000 rpm for 15 min, check on level: if ~500 μL is reached then top it up to 4 mL again either with elution or with phosphate buffer after using elution up completely
- 4 times 4 mL→ 500 μL should be done at least with phosphate
- Spin down for the last time to 200-500 μL and get it with gel-filling pipette tip (and a 200 μL pipette) first sucking up the yellow material (concentrated FAD-binding protein part) and then the rest, put it in a 1.5 mL Eppendorf tube that it going to be frozen in liquid N_2 and placed in the -80 ^oC box for storage and further usage
- Store all tubes on ice outside of the centrifuge
- Collect and store at 4 ^oC the filtrates until SDS-PAGE confirms protein

6. HIVATKOZÁSOK

Ambrus A, Torocsik B, Adam-Vizi V, Periplasmic cold expression and one-step purification of human dihydrolipoamide dehydrogenase. *Protein Expr. Purif.*, 63(1): 50-57 (2009)

7. MELLÉKLETEK ADATLAPOK JEGYZÉKE

Nincsennek mellékletek.