TARDIGARD

p r o t o c o l

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Preparing cell culture for protein expression

Day one

Extract target plasmids pMD19-Ptet-tetR-TDP-T3， as well as pMD19-Ptet-tetR-blank-T3—plasmid without protein that act as a control group

Transform it into competent cell MG1655

Grow overnight

Induction of TDP expression

Day two

Pick 3 colonies from each clone and inoculate it in 4ml LB + Amp in shaking tube; let them grow at 37 °C, 220 rpm 5h

At the same time, pre-heat the liquid medium at 37C so bacteria can directly go into log phase after dilution

Measure OD600, dilute the culture to OD600=0.2, then dilute in ratio of 1:1000

Incubate at 37 °C, 220 rpm 2h, dilute for the second time in ration 1:1000

Induce the culture with anhydrotetracycline of different concentration

0ng/ml, 2ng/ml ,4 ng/ml,6ng/ml,8 ng/ml,10ng/ml ,50 ng/ml ,100 ng/ml

anhydrotetracycline (ATc) is a tetracycline analog, showing increased (30x) affinity for tet repressor. 42 ng/mL gives induction at half maximum according to an in vitro assay

grow over night at 37 °C, 220 rpm

Protein Assay

Day three

Centrifuge the cell culture in 2ml centrifuge tube

Discard the supernatant

Wash with 1xPBS pH=7.4

Add cell lysis buffer to 1ml

Mix thoroughly with vortex

Pre-set up of centrifugal machine to 4°C

Cool the suspension on ice prior to sonication for at least 10min

Sonication

1. Wash the needle with ddH2O (precooling on ice)
2. To reduce the building up of heat which may denature proteins, performed on ice and sonicate intermittently
3. Amplitude：65%
   Pulse-ON: 10s
   Pulse-OFF: 5s

Centrifuge: speed 10 15min at 4°C

Collect the supernatant into 1.5 ml centrifuge tube

Protein Assay with BCA (see instruction in kit)

SDS-Page

An SDS-Page separate protein in a sample by weight

We will be looking for a protein band at expected size for CAHS and Dsup

CAHS: (699/3)*110=25630 (703/3)*110=25776 Dsup: (1351/3)*110=49536 (approx. 25kD and 50kD)

Gel concentration: 10% 2ml stacking gel and 5ml separating gel

Sample Preparation

1. Buffer with denaturant diluted with sample (dilute x5)
2. Heat 95°C for 5 minutes

Gel Preparation

1. Put two gel glass pieces together in the correct orientation and clip into the gel apparatus
2. Add water to test for leakage
3. Add about 5 mL of separating gel in between the two glass pieces (ensure it does not leak)
4. Add a thin layer of ddH2O on top of the separating gel to form a smooth line
5. Wait for the gel to solidify (in 37c indubator~ 20 minutes)
6. Pour out water
7. Add about 2 mL of the stacking gel on top of the separating gel and add the gel comb
8. Wait for the gel to solidify (~ 20 minutes)
9. Place the gel into the gel running apparatus and lock it in place. Ensure the side with the larger piece of glass is facing outward. **If there is only one gel, add empty glass pieces to the other side**

Run

1. Put the gel into the gel box and fill with 1x SDS Running Buffer. Ensure the middle section between the gels is filled with buffer and the
2. Load samples (20ug)
3. Run gel at 180V until samples leave the well and form a line at the boundary of two gels, increase the voltage to 100V. Or run the gel at lower voltages (~90V) to get cleaner bands

Stain and Destain

- Stain the gel with Coomassie Brilliant Blue for 1h
- Destain the gel with water