6xhis Lyticase Purification Protocol

pEL765	pT22- Lyticase no tag	AmpR
pEL766	pT22- 6HisLyticase	AmpR

Plasmid received from John Denu Lab, instructions from Slava

Media:

2x 2L of TB + 1L TB
Autoclave
Cool + add appropriate antibiotics

DAY 1

Transforming BL21-DE3 (RIPL) competent cells

1. Set heat block to 42ºC
2. Thaw BL21-DE3 (RIPL) cells ON ICE (Cells received from Mike Airola)
3. Prechill N+1 eppendorf tubes on ICE (N is the # of DNA samples to transform)
4. Label tubes, 1 for NO DNA control
5. Transfer 50-100uL of BL21-DE3 (RIPL) cells to pre-chilled tubes
6. Add DNA to cells and incubate on ice for 30 minutes [1uL DNA per 50 uL competent cells] → use pEL766 for nickel column purification
7. To no DNA control- add 1-7uL sterile H2O
8. Heat shock mixture for 45 seconds @ 42ºC
9. Incubate on ICE for 2 min
10. Add 10x volume [510 uL] of 2xYT or SOC. Mix well by inverting.
11. Incubate cells at 37ºC for 45-60 minutes on heat block
12. Collect cells by spinning at 9,000 rpm, 1 minute
13. Remove supernatant, leaving ~100uL behind
14. Resuspend cell pellet with remaining ~100uL media
15. Plate cells on LB+Amp plate, spread using glass beads. Leave upright until dry.
16. Incubate upside down at 37ºC in bacteria incubator overnight

Day 2

Expression

17. Inoculate BL21-DE3 (RIPL) transformants from step 15 into 50 mL of TB +Amp
    a. If you have hundreds of transformed colonies, you can perform induction in the same day
    b. Add 1-2 mL of TB (or LB or SOC - NO Amp) to each plate.
    c. Use a cell scraper to resuspend all the cells (pipette to resuspend very well)
    d. Transfer to eppendorf tube
18. Take the OD of plates by transferring 20 uL of cell suspension into 180 uL of the same media (TB) and measure OD
    a. yEL766 OD (1/10) = ___
19. If the final OD of suspension is > ~10 OD in total, transfer the entire suspension into 500 mL of the TB +Amp (500 uL amp in 500 mL TB) for growth at 37 ºC
    a. If the 1-2mL suspension is much less than 10 OD, then transfer the entire suspension into 50 mL of TB+Amp and allow growth at 37ºC for 2 hours, measure cell OD, transfer appropriate volume (or entire 50 mL culture) to make 500mL TB +Amp to make up ~0.02 OD. Continue the same.
20. Grow culture to 0.5-0.8 OD

Time	OD600 of pEL766 culture

    a. Remove ~500 uL of pre-IPTG sample. Spin 13,000 RPM for 1 min, discard supernatant, resuspend with 20uL 1x SDS+ BME
21. Add IPTG (233.5 uL for 500 mL) to induce protein expression
22. Switch incubators temp to 25ºC and incubate overnight

DAY 3

Harvest cells

Time	OD600 of pEL766 culture

Remove 500uL equivalent from the previous day, using OD to normalize. Label tube after protein induction. Spin down cells. Resuspend in 1xSDS+BME and save for induction analysis.

23. Pellet cells at 4,000 rpm for 5 minutes
24. Wash pellet with H2O
25. Resuspend in ice cold H2O
26. Spin down, remove supernatant, flash freeze in 50 mL conical tube and store in -80ºC.
    a. Saved in 2 pellets: 250 mL equivalents because of growth

Analyze Protein Induction

27. Thaw -IPTG and +IPTG cells. Increase volume to ~100 uL with 1xSDS+bME.
28. Sonicate samples @ 60 Hz for 1 min 30 sec (15 S-ON, 15 S-OFF)
    a. Place samples in a turning rig, parafilm over the top to stop water from coming in.
    b. Plug holes in sonicator with parafilm and fill with water (Does not need to be cold)
    c. Attach power to bottom
    d. Set up the program on the machine to the right, use the non-precision tip.
29. Heat samples @ 95ºC for 5 minutes
30. Centrifuge samples at 13,000 rpm for 5 minutes
31. Load 40 uL of samples into 8-16% SDS-PAGE . Run 150V for 1 hr and 25 min
32. Stain Sypro Orange (5uL stain in 50 mL 7.5% Acetic Acid) for 30 minutes on rocker
33. Rinse 2x with 7.5% Acetic Acid for 15 minutes
34. Scan to identify if Lyticase has been overexpressed. Expected MW = ~55 kDa
    a. Protein induction is not obvious.

[Plasmid DNA isolation from yeast using the QIAprep SPin Miniprep Kit was performed 1.13.22]

Day 4

Sonicate pEL766 (6xHis Lyticase) Pellet

1. Make Standard sonication Buffer and chill on ICE
    a. 50 mM Tris-HCL (pH7.6), 10 mM Imidazole, 500 mM NaCl, 3 mM bME, 0.2mM PMSF
    b. Use ~30 mL sonication buffer per 250 mL equivalent
2. Retrieve cell pellet from -80ºC and thaw at RT for 10-15 minutes
3. Pre-chill 50 mL glass beaker

4. Resuspend pellet in 30 mL of sonication buffer. Transfer the suspension into the chilled beaker.
5. Place the beaker in Ice water bath for sonication.
6. Sonicate cells using Microtip (short stubby one) @ 50Hz for 12x15 sec pulses (15 sec ON, 30 sec OFF)
    a. Place tip so that it is in the middle of the beaker to avoid bubbles
7. Remove 50 uL sample and label it sonicated WCE
8. Centrifuge sample in JA-25.50 rotor at 15,500 rpm for 20 min @ 4ºC
    a. Prechill 50mL conical tube
9. Transfer supernatant to the pre-chilled 50 mL conical tube
    a. Remove 1mL for test TALON bead pulldown

Pilot TALON Pulldown

10. Remove 50 uL for gel, label sonicated cleared WCE
11. Take 100 uL slurry volume TALON beads, spin down beads, wash with sonication buffer 2x
12. Resuspend beads with 1 mL sonicated WCE
13. Incubate in cold room on rocket 30-60 min
14. Spin down beads, collect 50 uL supernatant and label after TALON binding
15. Elute Lyticase from Talon beads with 1 mL of Buffer with 300 mM imidazole
    a. Incubate for 15 minutes
16. Spin down beads, remove 50 uL elution, label elute
    a. Save supernatant
17. Flash Freeze and store samples in -20ºC until ready to analyze on SDS

Analyze TALON pulldown Pilot

18. Remove samples from -20ºC
    a. pEL766 WCE -IPTG
    b. pEL766 WCE +IPTG
    c. After sonication WCE
    d. Sonicated- cleared WCE
    e. Sup't after TALOT pulldown
    f. Elution of Lyticase
19. Add 20 uL of 2xSDS+bME to 20 uL samples a-f (+ kaleidoscope ladder with 2xSDS-bME)
20. Heat samples @ 95ºC for 5 minutes, spin down
21. Load 40 uL of each sample onto 8-16% SDS PAGE, Run at 15-V for 1hr 30min
22. Stain with SYPRO ORANGE 1:10,000 in 7.5% Acetic Acid 30 min
23. 2x 15 min wash with 7.5% Acetic Acid
24. Identify band at ~55kDa in Elute

Day 4 or 5

First step in purifying 6xhis Lyticase with FPLC (HisTrap HP)

1. Make 500 mL Buffer A and Buffer B, filter sterilize, chill

Buffer A: 50 mM Tris HCl (pH 7.6), 500 mM NaCl, 3 mM bME, 0 mM Imidazole
Buffer B: 50 mM Tris HCl (pH 7.6), 500 mM NaCl, 3 mM bME, 500 mM Imidazole
Buffer S1: 50 mM Tris HCl (pH 7.6), 500 mM NaCl, 3 mM bME, 10 mM Imidazole

2. Install HisTrap HP 1 mL in V9-2
3. Use program: CC210119_TALON_Superflow_Gradient_Lyticase_purification
4. Wash and prep all tubing (A, B, S1, Buffer)
5. Place S1 tubing in 30 mL filter sterilized lysate (tape down tubing to the bottom)
    a. Cindy Book #4, pg 170 for example picture
6. Save, collect and analyze 50 uL of input, outlet 6 (FT) and peak fractions
    a. Add 20 uL 2xSDS + bME to 20 uL sample
    b. Heat samples at 65ºC, 5 min, spin down
    c. Load 40 uL into 6-8% SDS PAGE
    d. Run 150 V for 1hr 40min
    e. Stain SYPRO ORANGE
7. Identify fractions containing 6xhis lticase (Expected in fractions 14-22)

Second step in Purifying 6xhis Lyticase with FPLC (SP Column)

Exchange buffer:

Glucanase has a PI of ~6, making it relatively neutral. The current buffer it is in is pH 7.6, resulting in a partial negative charge. However, we used a Q column and that didn't bind. Use size exclusion column (pd10) to exchange Tris pH7.6 for MES pH 6 buffer and desalt before binding to an SP column.

Elution buffer: 2mM Mes pH 6, 10% sucrose, 10% glycerol

1. Concentrate Lyticase expected fractions (14-22) to 2.5 mL
2. Prepare PD-10 Desalting column by removing the top cap and pouring off the column storage buffer and cutting the sealed end of the column at the notch.
    a. Clamp column to pole in cold room
3. Equilibrate column by filling the column with an equilibration buffer and allow the equilibration buffer to enter the packed bed completely.

    a. Repeat 4x or use a funnel cap to a volume of 25 mL. Discard the flow through.
4. Apply 2.5 mL of sample to the column and allow it to enter the packed bed completely. Discard flow through.
5. Place a test tube for sample collection under the column
6. Elute with 3.5 mL buffer and collect the elute.

Prepare SP column for use

Buffer A: 2mM Mes pH 6
Buffer B: 25mM Mes pH 6, 750mM NaCl, 1mM EDTA
Use program: CC210120_TALON_Superflow_cation_Lyticase_purification

1. Save, collect and analyze 50 uL of input, outlet 6 (FT) and peak fractions
    a. Add 20 uL 2xSDS + bME to 20 uL sample
    b. Heat samples at 65ºC, 5 min, spin down
    c. Load 40 uL into 6-8% SDS PAGE
    d. Run 150 V for 1hr 40min
    e. Stain SYPRO ORANGE
2. Identify fractions containing 6xhis lticase (Expected in fractions 14-22)

Perform Activity Assay on Fractions in Question

1. Concentrate peak fractions from Lyticase FPLC from ~9 mL to ~500 uL
    a. Taking individual fractions is not concentrated enough to see spheroplasting.
2. Thaw cell pellet (400 mL eqv) from -80ºC (I used yEL152)
3. Resuspend in 6 mL of PS Buffer per 400 mL equivalent, add 6uL DTT
4. Incubate at room temperature for 10 minute
5. Spin at 3K rpms for 5 min in SX-4750A rotor. Discard supernatants.
6. Resuspend pellet in 4.5 mL S buffer (per 400 mL equiv), add 4 uL DTT
7. Split into 5x 1mL fractions in eppendorf tubes labeled 10, 20, 30, 40 and 50
    a. Save 10 uL each for A600 analysis time 0
8. Add 10uL, 20 uL, 30 uL, 40 uL and 50 uL of concentrated Lyticase into their respective tube with cells. Mix well. Label tubes.
9. Incubate at 30ºC (use water bath on my bench, fully submerge)
10. Take OD600 reading every 15 minutes by mixing 10 uL cells + 190 uL water
11. If the OD decreases substantially over the hour, you have Lyticase and can move on.

	T= 0 min	15	30	45	60
10					

20					
30					
40					
50					

Dialyze into Storage buffer

- Containing 10% glycerol, 5mM DTT,

Stripping and Recharging HisTrap HP 1mL column

Stripping

Stripping buffer: 20 mM Sodium Phosphate, o.5M NaCl, 50 mM EDTA, pH 7.4

1. Strip the column by washing with at least 5-10 column volumes of stripping buffer.
2. Wash with at least 5-10 column volumes of binding buffer
3. Wash with at least 5-10 column volumes of distilled water before recharging the column.

Recharge

1. Re-charge the water-washed column by loading 0.5 mL of 0.1 M $NiSO_4$ in distilled water for 1 mL column (or 2.5 mL for 5 mL column).
2. Wash with 5 column volumes distilled water
3. Wash with 5 column volumes binding buffer