RECOMBINANT PNEUMOLYSIN – EXPRESSION, PURIFICATION AND RED BLOOD CELL LYSIS

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BACKGROUND

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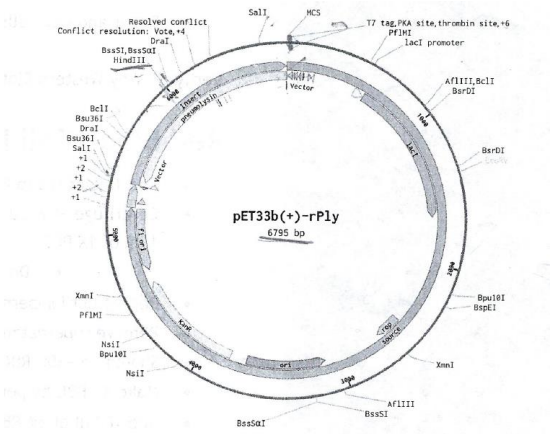
NOTES

- The plasmid **pET33b(+)-rPly** was sequenced and kindly provided by the Orihuela lab (University of Alabama at Birmingham, AL, USA)



Plasmid map from sequenced expression vector. Sequenced by Orihuela la
April 2018 (Heflin Genomic Center, UAB)

- The plasmid, encoding **His-tagged *S. pneumoniae* pneumolysin and a Kanamycin resistance gene**, was transformed into *E. coli* BL21(DE3)
- freezer stocks of this strain are stored **in the Bowdish Lab -80 °C freezer**; Box "Plasmids in *E. coli* DH5α" (please see -80 °C Freezer and "Bowdish Plasmid" inventory)
- Before starting the pneumolysin expression it is good microbiological practice to **streak out the *E. coli* BL21(DE3) pET33b(+)-rPLY on LB agar plates, containing 50 µg/mL Kanamycin**, and incubating over night at 37 °C; the plate can then be stored in the fridge for a couple of weeks
  → Pick a few bacterial colonies with an inoculation loop from the plate and inoculate a starting culture (see section "1. Pneumolysin expression")

EQUIPMENT

Pneumolysin expression:

- *E. coli* BL21(DE3) pET33b(+)-rPly (stored in -80 °C freezer)
- LB medium
- Kanamycin (stock solution 50 mg/mL)
- IPTG
- Sterile Erlenmeyer flasks

Cell disruption and pneumolysin purification:

- 500 mL Equilibration buffer (PBS containing 10 mM Imidazole; sterile filtered through 0.45 µm filter)*
- Elution buffer (PBS containing 250 mM Imidazole, sterile filtered through 0.45 µm filter)*
- 70 % EtOH
- Cell disrupter (CMCB, McMaster University)

*buffers are for any His-tagged protein; more suitable buffers can be tested

SDS PAGE and Dialysis

- 40 % Acrylamide/Bis
- 1.5 M Tris-HCl
- 0.5 M Tris-HCl
- 10 % SDS
- TEMED
- 10 % APS
- SDS running buffer
- Coomassie staining
- De-stain solution
- Loading buffer
- Protein ladder
- Dialysis buffer (20 mM HEPES, 150 mM NaCl, pH 7.5; filtered through 0.45 µm filter)

Red blood cell hemolysis to determine hemolytic activity

- Sheep blood
- 96-round bottom plate, 96-well flat bottom plate
- 1X PBS
- Plate Reader

PROTOCOL

DAY 1 and DAY 2

1. **Pneumolysin expression:**
   - Culture *E. coli* BL21(DE3) pET33b(+)-rPly from an LB Kan 50 plate in 10 - 20 mL LB medium containing 50 µg/mL Kanamycin
     → overnight, 37 °C, 225 rpm
   - Determine the OD_{600} of the overnight culture
   - Start a large volume of expression culture (250 – 500 mL; I used 500 mL which resulted in approximately 20 mL of protein)
     → Dilute the overnight culture in fresh LB medium (50 µg/mL Kanamycin) to an OD_{600} = 0.05

→ culture at 37 °C, 225 rpm until OD_{600} = 0.4 – 0.8 (I used 0.4)
→ After reaching OD_{600} = 0.4, induce pneumolysin expression by adding IPTG (final concentration 0.5 mM) to the culture
→ Grow the culture for another 3 h at 37 °C, 225 rpm

- Pellet the entire culture by centrifugation at 4 000 rpm for 10 min, 4 °C (in 50 mL tubes)
- Discard the supernatant and freeze the pellet at -80 °C until purification step

2. <u>Preparation of SDS gels</u>

- Prepare at least two SDS gels and store under humid conditions in the fridge until usage
- **Recipe is for 2 gels:**

a) <u>Resolving Gel</u>

Component**	**Volume**
40 % Acrylamide/Bis	4.5 mL
1.5 M Tris-HCl	3.75 mL
10 % SDS	150 µL
dH_2O	6.5 mL
TEMED	7.5 µL
10 % APS in dH_2O*	75 µL

*Prepare 10% APS fresh in dH_2O
**Add TEMED and 10% APS only when you are ready to pour the gel

- Gel need approximately 45 min to solidify at room temperature

b) <u>Stacking Gel</u>

Component**	**Volume**
40 % Acrylamide/Bis	1.5 mL
0.5 M Tris-HCl	3.75 mL
10 % SDS	150 µL
dH_2O	9.5 mL
TEMED	15 µL
10 % APS in H_2O	75 µL

*Prepare 10% APS fresh in dH_2O
**Add TEMED and 10% APS only when you are ready to pour the gel

- Gel need approximately 45 min to solidify at room temperature

- Wrap gels in with H_2O soaked paper towels, put them in a plastic bag and store them at 4 °C in the fridge until usage

DAY3

3. <u>Cell disruption and protein purification:</u>

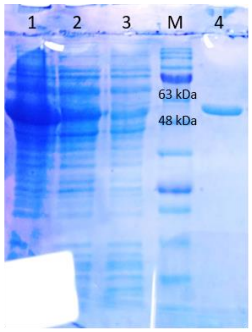
- Resuspend cell pellet in 20 mL (if you had a 500 mL culture) Equilibration buffer and place on ice
- **OPTIONAL:** add protease inhibitor that doesn't contain EDTA, 1 mM PMSF would work
- Disrupt cells using cell disrupter (CMCB, McMaster University, person to contact Tracey Campbell)
  → cell disrupter holds a volume of 10 mL
  → unlock cell disrupter
  → run H_2O first to make sure it is clean, put some lubricant on O-ring, put pieces of cell disrupter together

→ Set pressure to 20 Psi, press Start
→ Remove H_2O and proceed with sample in the same way
→ if sample volume is bigger than 10 mL, repeat the procedure

- Collect disrupted cells in 50 mL tube, place on ice
  → take 30 µL of disrupted cells and store on ice until SDS PAGE **(whole cell lysate)**
- Clean the cell disrupter: Spray everything with EtOH and rinse with H_2O
  → Run water through it as at the beginning
- Spin down the cell debris at 10 000 – 13 000 rpm for 30 min at 4 °C
  → Transfer 30 µL of supernatant into 1.5 mL tube and store on ice until SDS PAGE **(protein loaded on column)**
  → transfer the supernatant to a fresh 50 mL Falcon tube
- Purify protein with His-Trap column (5 mL) via FPLC at 4 °C (CMCB, McMaster University, person to contact Tracey Campbell)
  → Collect Flow through in 50 mL tubes
  → collect 1 mL fractions in 96-well plates
  → Transfer 30 µL of column flow through into 1.5 mL tube and store on ice until SDS PAGE **(Flow through)**
  → keep fractions of purified protein at 4 °C

4. <u>SDS PAGE and Dialysis:</u>

- Check FPLC spectrum for protein peak and select multiple fractions from the beginning to the end of the peak for SDS PAGE
- Mix 2 µL of 6x Loading buffer with 10 µL of samples and load everything onto SDS gels
- Run the gel at 200 V for approx. 45 min at room temperature
- Take out gels put them individually in boxes, rinse with H_2O and add Coomassie staining
  → approx. 2 h at room temperature
- Discard Coomassie and rinse gel with H_2O
- add De-staining buffer for approximately 1.5 h at room temperature
- Discard the De-stain buffer and add H_2O to the gels



1) Cell lysate (lysed but not spun down)
2) Column (lysed and spun down, loaded on column)
3) Column Flow through
M) Protein ladder
4) Fraction F2

Pneumolysin 53 kDa

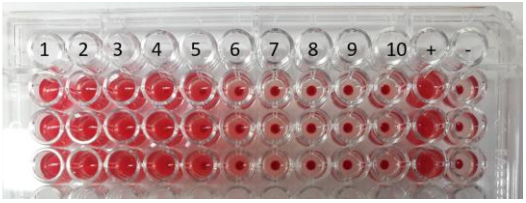
- Pool the fractions that contain only pneumolysin (53 kDa) into a new 50 mL tube
- Dialyze the protein in 4 L of dialysis buffer (contains less salt than the Elution buffer)
  → overnight at 4 °C, stirring at low rpm
- Remove the dialyzed protein from the dialysis device
- Either make small Aliquots (20, 50 or 100 µL) and store them at -80 °C for long term storage or directly perform hemolysis assay before making aliquots
- **A box with Pneumolysin aliquots is stored in the-80 °C freezer (see inventory) and a few aliquots are stored also at -20 °C**

DAY 4

5. **Hemolysis activity assay:**

- Add 1 mL of sheep red blood cells (RBCs) to a 1.5 mL tube
- Centrifuge at 2000 rpm for 5 min at room temperature
  → remove supernatant and add 1 mL of 1x PBS (DO NOT remove RBCs)
- Repeat until supernatant is completely clear (5 – 6x)
- Remove supernatant and add 1mL of 1x PBS
  → should be ~ 30 % RBCs
- Dilute RBCs 1:10 with 1x PBS to get ~ 3 % RBCs
- Add 100 µL of 3 % RBCs to wells of 96-well round bottom plate
- Make pneumolysin dilutions of interest (e.g. 1:2) and add 100 µL to the wells
  → I had 50 % activity at a 1:1000 dilution
- Include controls: 100 % lysis (1% Triton in dialysis buffer)
  0 % lysis (1x dialysis buffer)
- Incubate at 37 °C for 30 min
- Centrifuge plate at 1 500 rpm for 10 min at room temperature
- Transfer 100 µL of supernatant to wells of a 96-well flat bottom plate
- Measure the OD_{540} of the supernatant and determine the hemolytic activity

Example:



1) Undiluted pneumolysin
2) 1:10
3) 1:100
4) 1:500
5) 1:1000
6) 1:2000
7) 1:4000
8) 1:8000
9) 1:16 000
10) 1: 32 000
+) Ctrl. 100 % Lysis (1% Triton)
-) Ctrl. 0 % Lysis (buffer)

ANALYSIS/SOFTWARE

- Plate reader
- 1 hemolytic unit is LD_{50} (concentration at which 50 % of blood cells lyse)

LINKS AND REFERENCES

- Protein expression, purification and hemolysis protocol was provided by Orihuela Lab (University of Alabama at Birmingham, AL, USA)
- **Publications about Pneumolysin purification, dialysis, hemolysis assay:**
  - Marini et al. Experimental design approach in recombinant protein expression: determining medium composition and induction conditions for expression of pneumolysin from *Streptococcus pneumoniae* in *Escherichia coli* and preliminary purification process. **BMC Biotechnology 2014: 9;14:1, doi: 10.1186/1472-6750-14-1.**
  - Lawrence et al. Crystal structure of *Streptococcus pneumoniae* pneumolysin provides key insights into early steps of pore formation. **Sci Rep. 2015, 25;5:14352. doi: 10.1038/srep14352.**