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6L Live PlmCasX Expression & Purification

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ABSTRACT

This protocol describes methods to express and purify PlmCasX plasmids.

PlmCasX_Live_6L_ExpressionPurification_Protocol.pdf

GUIDELINES

Perform all bacterial culture steps aseptically.

MATERIALS

NAME	CATALOG #	VENDOR
DNase I (RNase-free) - 1,000 units	M0303S	New England Biolabs
LB Broth	10855001	Thermo Fisher
Ampicillin, sodium salt, irradiated	11593027	Thermo Fisher
IPTG	15529019	Thermo Fisher
Terrific Broth	A1374301	Thermo Fisher
J6-MI High-Capacity Centrifuge 60 Hz	360291	Beckman Coulter
Pyrex® baffled shaker flasks	CLS44441L	Sigma Aldrich
Avanti® J-E Centrifuge 50/60 Hz	369001	Beckman Coulter
TEV protease	P8112S	New England Biolabs
Amicon® Ultra-15 Centrifugal Filter Units	UFC905008	Emd Millipore
Amicon® Ultra-4 Centrifugal Filter Units	UFC803008	Emd Millipore
HiTrap SP HP 5 x 5 ml	17115201	Ge Healthcare
cOmplete™ Protease Inhibitor Cocktail	11697498001	Sigma Aldrich
PMSF (Phenylmethylsulfonyl fluoride)	10837091001	Sigma Aldrich
Corning® 150 mL Bottle Top Vacuum Filter 0.22 µm Pore 13.6cm² PES	431161	Corning
Ni-NTA Agarose	30210	Qiagen
Econo-Column Chromatography Columns 1.5 × 10 cm	7371512	Bio-rad Laboratories
Misonix Inc S-3000 Misonix Sonicator 3000 Ultrasonic Cell Disruptor with Temperature Control	EW-04711-81	Cole Parmer
Rosetta™(DE3) Competent Cells - Novagen	70954	Emd Millipore

SAFETY WARNINGS

Please refer to Safety Data Sheets (SDS) for health and environmental hazards.

Day 1: Grow pre-culture

1 Set up pre-culture by either **transforming plasmid into Rosetta cells** or using a small amount from a **glycerol stock** (steps found below).

Perform all steps aseptically, next to a Bunsen burner.

step case

Set up pre-culture (transform plasmid)

Aseptically transform plasmid into competent Rosetta cells

1. Thaw 10 µl competent Rosetta cells on ice, flick tube to mix
2. Add 1 µl ~200 µM plasmid to cells and rest on ice for 5 – 10 minutes
3. Heat shock cells in 42 °C water bath for 35 – 45 seconds
4. Add 300 µl room temperature Luria-Bertani (LB) broth to tube and rest on ice for 2 minutes
5. Shake cells at 37 °C, 160 – 180 rpm for 1 hour

2 

Add **200 ml LB broth** to a 500 ml baffled Erlenmeyer flask.

3 

Add Ampicillin to a final concentration of **50 milligram per liter (mg/L)** (add **200 µl** of **50 milligram per milliliter (mg/mL)** Ampicillin).

4 

Add the entire tube of transformed cells to flask.

5 

Shake at **37 °C** , **160 rpm** – **180 rpm** , **Overnight** .

Day 2: Grow TB culture

6

Locate 6 glass or plastic baffled 1 l flasks and add **1 L TB broth** to each flask.

Perform all steps in this section aseptically, next to a Bunsen burner.

7 

Add Ampicillin to a final concentration of **50 milligram per liter (mg/L)** (add **1 ml** of **50 milligram per milliliter (mg/mL)** amp per flask).

8 

Add **20 ml** – **25 ml** of pre-culture (grown in LB broth) to each flask of TB broth.

9 

Shake at **37 °C**, **160 rpm** – **180 rpm** until the **OD600** measurement is between **0.5 – 0.6** (roughly 3 – 6 hours).

Be sure to blank spectrophotometer with cuvette of clean media.

Day 2: Induce protein expression

10 Transfer TB flasks to be **On ice**.

11 

Add IPTG to a final concentration of **1 Milimolar (mM)** to each flask (**1 ml** **1 Molarity (M)** IPTG per flask).

12 

Shake at **16 °C**, **160 rpm** – **180 rpm**, **Overnight**.

Day 3: Lyse bacterial cells

13 Prepare Lysis Buffer + TCEP (final volume 160mL):

The recipe for Lysis Buffer per 20mL of volume is:
1 Roche cOmplete protease inhibitor tablet
2uL DNase (RNase free)
0.5mM PMSF final concentration

13.1 Add 8 Roche cOmplete protease inhibitor tablets.

13.2 Add **16 µl** DNase (RNase free).

13.3 Add PMSF to a final concentration of **0.5 Milimolar (mM)** (**800 µl** of **100 Milimolar (mM)** stock PMSF).

13.4 Add TCEP to a final concentration of [M]**0.5 Milimolar (mM)** (**80 µl** of [M]**1 Molarity (M)** stock TCEP).

13.5 Fill to final volume of 160mL using MilliQ water.

14 Transfer cultures to 1L plastic centrifuge tubes and balance pairs of tubes by adding MilliQ H2O.

15 

Centrifuge cultures in a Beckman Coulter J6-MI centrifuge at **4000 rpm** , **00:20:00** , **4 °C** .

Use blue sleeves to ensure tubes fit snuggly.

16 Pour off supernatant from cultures.

17 

Add **20 ml** Lysis Buffer + TCEP to each plastic tube.

18 Vortex tubes until sample is homogenous.

Use a flat-top vortex.

19 

Transfer cell suspension to a 500 ml beaker.

Alternatively, lysed cells can be frozen down for later use. Transfer to 5mL tubes and centrifuge at 4,000rpm, 20 minutes, 4°C. Pour off supernatant and freeze pellet at -20°C.

20 

Add solution of tablets + PMSF + DNase to cell suspension.

21 Place sample in tube of ice with water to hold beaker snugly.

22 Sonicate sample in cold room.

22.1 Rinse tip of sonicator with ethanol, then H_2O.

22.2 Set sonicator to run at 5.5 power setting for **00:05:00** , cycling between **00:00:10 on** and **00:00:15 off** .

23 Place 6 plastic ultracentrifuge tubes (with caps) **On ice** .

24 Divide sonicated sample between ultracentrifuge tubes. Use scale to match pairs within +/- 0.01 g by adding MilliQ H2O.

25 

Centrifuge samples in Beckman Coulter Avanti J-E centrifuge at **35000 rpm** , **00:45:00** , **4 °C** .

26 Filter sonicated sample through bottle top **0.22 µm** filter and store **On ice** .

Day 3: Ni-NTA column binding

27 

Add **20 ml** Qiagen Ni-NTA resin to yellow gravity column (Econo-Column Chromatography Column) with butterfly valve in **4 °C** cold room.

28 Drain off ethanol storage buffer.

29 

Wash with **10 ml** Lysis Buffer + TCEP. (Repeat 4 more times).

29.1 Wash with **10 ml** Lysis Buffer + TCEP.

29.2 Wash with **10 ml** Lysis Buffer + TCEP.

29.3 Wash with **10 ml** Lysis Buffer + TCEP.

29.4 Wash with **10 ml** Lysis Buffer + TCEP.

30 

Add **10 ml** Lysis Buffer + TCEP to Ni-NTA column, mix with pipette.

31 

Transfer Ni-NTA resin to bottle with sonicated/filtered sample and add stir bar.

Do not discard yellow gravity column, it will be used again in step 33.

32 Stir sample in Ni-NTA resin at **4 °C** for at least **02:00:00** .

33 Add sample in Ni-NTA resin back to yellow gravity column.

34 Drain liquid.

Collect flow-through for gel electrophoresis.

35 

Add **50 ml** Lysis Buffer + TCEP.

36 Drain liquid.

Collect flow-through for gel electrophoresis.

37 

Add **10 ml** Lysis Buffer + TCEP.

38 

Add **2 milligram per milliliter (mg/mL)** TEV protease (4 eppendorfs of **250 µl** lab stock).

39 Mix sample in Ni-NTA resin well.

40 

Incubate column at 4 °C , Overnight .

Day 4: Elution of Ni-NTA column

41 Elute Ni-NTA column into 50 ml Falcon tube. Keep On ice . Label as “flow-through.”

42 

Wash column with 10 ml Elution Buffer ([M]500 Milimolar (mM) **imidazole**). (**Repeat** this step **2 more times**).
Collect samples into 50 ml Falcon tubes and label with Elution numbers (I.e. label elution #1 this step, elution #2 next, etc.).

42.1 Wash column with 10 ml Elution Buffer ([M]500 Milimolar (mM) **imidazole**).

42.2 Wash column with 10 ml Elution Buffer ([M]500 Milimolar (mM) **imidazole**).

43 Pool fractions into 15 ml 50 kDa MWCO Amicon Concentrators.

44 

Centrifuge at 4000 rpm , 4 °C until volume is less than 4 ml .

45 Transfer to 4 ml 30 kDa MWCO Amicon Concentrator.

46 

Centrifuge until volume is less than 500 µl .

Day 4: SP Column Ion Exchange

47 Prepare IEX Buffer A, using MilliQ water as solvent:

47.1 Add [M]50 Milimolar (mM) HEPES (pH7.5).

47.2 Add [M]500 Milimolar (mM) NaCl.

47.3 Add [M]10 % volume glycerol.

47.4 Add TCEP to final concentration [M]**0.5 Milimolar (mM)** .

48 Prepare IEX Buffer B, using MilliQ water as solvent:

48.1 Add [M]**50 Milimolar (mM)** HEPES (pH**7.5**).

48.2 Add [M]**2 Molarity (M)** NaCl.

48.3 Add [M]**10 % volume** glycerol.

48.4 Add TCEP to final concentration [M]**0.5 Milimolar (mM)** .

49 Run Äkta at **0.25 ml/min** to attach column.

Use a **5 ml SP Column**, Screw the bottom of column into top Äkta port, making sure to use wet connections. Use pre-column pressure alarm set at **0.5 MPa**.

50 Equilibrate column with **40 ml** IEX A Buffer.

Run **5 – 10 Column Volumes** at **1 – 5 ml/min**, using pre-column pressure alarm set to **0.5 MPa**.

51 Run Äkta at **0.25 ml/min** to detach column.

52 Draw up **500 µl** sample with a 1 ml syringe.

53 Attach threaded luer lock attachment and remove air from syringe.

54 Manually inject sample into top of 5 ml SP column.

Use wet connections and **don't exceed 5 ml/min**.

55 Run Äkta at **0.25 ml/min** to attach column.

56 Insert new 96 deep well plate into **position 3**.

57 Run SP column gradient program between IEX Buffer A and B to elute.

58 Collect fractions from chromatogram peak. Concentrate fractions if necessary.

Save samples for gel electrophoresis.

Day 5: Size Exclusion Chromatography

59 Run Äkta at **0.25 ml/min** to attached S200 10/300 increase column.

Use 24 ml column, run at **0.5 ml/min**, with **2.8 MPa** pre-column pressure.

60 Add **500 µl** loop to Äkta.

61 Inject **3 ml** SEC Buffer into loop with 5 ml syringe with the red luer lock attachment.

62 Draw up sample into 1 ml syringe with the red luer lock attachment.

63 Inject sample (which should be less than **500 µl**) into loop, leaving syringe attached.

64 Run SEC program. The sample should be injected from the loop.

In System Control on the Akta software, go to the menu bar at the top of the page, select Method Editor > New. Under column type, select Gel Filtration. Choose 'Superdex 200 10/300 Increase' for the Column type.

65 Collect fractions from chromatogram. Concentrate as necessary.

Save samples for gel.

66 Nanodrop protein amount.

67 

Aliquot into tubes.

68 Flash freeze in liquid nitrogen and store at **-80 °C** .

Day 1: Grow pre-culture

step case

Set up pre-culture (take stab from glycerol stock)

Aseptically take a small amount of the glycerol stock and place directly into 300 µl Luria-Bertani (LB) broth

1. Shake cells at 37 °C, 160 – 180 rpm for 1 hour

2 

Add **200 ml** **LB broth** to a 500 ml baffled Erlenmeyer flask.

3 

Add Ampicillin to a final concentration of **50 milligram per liter (mg/L)** (add **200 µl** of **50 undefined**).