**Human PDE4D (86-413) Purification Protocol Draft**

**NEB 5-alpha Competent *E. coli* Transformation**

**[Simultaneously with BL21 Competent *E. coli* Transformation]**

* Chill two 14.0-mL transformation tubes on wet ice.
* Thaw a tube of NEB 5-alpha competent *E. coli* cells on wet ice until the last ice crystals disappear. Mix gently and carefully pipette 100.0-µL of NEB 5-alpha competent *E. coli* cells into transformation tube on ice.
* Add 2.0- µL of pET15B PDE4D (86-413) plasmid that was resuspended in TE buffer into first *E. coli* cell mixture transformation tube. Carefully flick tube 4-5 times to mix cells and DNA. Do not vortex.
* Add 2.0- µL of 50 pg/µL pUC19 Control DNA to second *E. coli* cell mixture transformation tube. Carefully flick tube 4-5 times to mix cells and DNA. Do not vortex.
* Place the mixtures on ice for 30 minutes. Do not mix.
* Heat shock at exactly 42°C in a water bath for exactly 30 seconds. Do not mix.
* Pipette 900-µL of room temperature SOC Outgrowth Media into the mixture.
* Place at 37°C for 1 hour. Shake vigorously (250 rpm) or rotate.
* Warm selection plates to 37°C.
* Mix the cells thoroughly by flicking the tube and inverting.
* On eight selection plates spread 100-µL onto each plate. Wait 10 minute before inverting. Incubate overnight at 37 °C. Alternatively, incubate at 30°C for 34-36 hours or 25°C for 48 hours.

**BL21 Competent *E. coli* Transformation**

**[Simultaneously with NEB 5-alpha Competent *E. coli* Transformation]**

* Thaw a tube of BL21 Competent *E. coli* cells on ice for 10 minutes.
* Add 1–5 μl containing 1 pg–100 ng of plasmid DNA to the cell mixture. Carefully flick the tube 4–5 times to mix cells and DNA. **Do not vortex.**
* Place the mixture on ice for 30 minutes. Do not mix.
* Heat shock at exactly 42°C for exactly 10 seconds. Do not mix.
* Place on ice for 5 minutes. Do not mix.
* Pipette 950 μl of room temperature SOC into the mixture.
* Place at 37°C for 60 minutes. Shake vigorously (250 rpm) or rotate.
* Warm selection plates to 37°C.
* Mix the cells thoroughly by flicking the tube and inverting, then perform several 10-fold serial dilutions in SOC.
* Spread 50–100 μl of each dilution onto a selection plate and incubate overnight at 37°C. Alternatively, incubate at 30°C for 20–24 hours or at 25°C for 48 hours.

**Growth of *E. coli* cell**

* Transfer one colony of *E. coli* strain BL21 (codonplus) transformed with the vector pET15b-PDE4D (86-413) into a 500ml LB culture medium (after autoclaved 10g bacto-tryptone, 5g bacto-yeast extract, 10g NaCl per liter, add 0.4% glucose, 100mg AMP, and 20mg chloramphenicol per liter LB).
* Grow cell at 37°C to A600 = 0.7 (about 8 hours). *Use* *LB media as the reference.*
* Once it reached the O.D. of 0.7 add 0.1 mM IPTG for further growth at 15°C for overnight. Typical yield will be about 7 g cell per liter culture. Harvested cells are centrifuged, weighed, and stored at -80°C for use.

**Extraction**

***(NOTE: A typical batch of purification uses 7 grams of cell from 1 liter culture.)***

* Add 4 ml of the extraction buffer per gram of frozen cells and homogenize it. *[The extraction buffer is 20 mM Tris.base, pH 8.0, 0.3 M NaCl, 15 mM imidazole, 1 mM β-ME plus inhibitors 0.2 mM PMSF, 0.2 µg/ml aprotinin, and 0.2 µg/ml pepstatin]*
* Pass the suspension through French Press three times at 1200 psi. **[SONICATE?? Amplitude 35% for 4-5 minutes – pulse 2 secs ON and 5 secs OFF (From Taylor Lab)]**
* Centrifuge at 15k rpm for 20 min in a JA20 rotor. Collect the supernatant.

**Ni-NTA column of His-tag PDE4D2**

* Load the supernatant into a Ni-NTA column (φ=2.5 cm, 25 ml QIAGEN agarose beads).
* Wash the column at 6 ml/min with three buffers that contains inhibitors of 0.2 mM PMSF, 0.2 µg/ml leupeptin, 0.2 µg/ml aprotinin, and 0.2 µg/ml pepstatin.
* 300 ml **buffer 1**, 20 mM Tris.base, pH 8.0, 0.3 M NaCl, 15 mM imidazole, 1 mM β-ME.
* 200 ml **buffer 2**, 20 mM malic acid, pH 6.5, 0.3 M NaCl, 15 mM imidazole, 1 mM β-ME.
* 100 ml **buffer 3**, 20 mM Tris.base, pH 8.0, 50 mM NaCl, 15 mM imidazole, 1 mM β-ME.
* Elute PDE4D2 out with 20 mM Tris.base, pH 8.0, 50 mM NaCl, 100 mM imidazole, 1 mM β-ME.
* Concentrate PDE4D (fractions 5-12) with Amicon YM30 to 10-20 ml.
* Dialyze against 1 liter 20 mM Tris.base, pH 7.5, 50 mM NaCl, 2.5 mM CaCl2, 1 mM β-ME for 1 hour.

**Thrombin Digestion**

* Combine PDE4D2 fractions 5-12, add 2mM CaCl2 5 λ 14 mg/ml thrombin (Haematologic Tech. Inc) and oscillate at room temperature for digestion of one hour.
* Add 1 mM EDTA to stop the cleavage.

**Q-Sepharose column**

* Load into a Q-sepharose column (φ2.5 x 8 cm).
* Wash the column with 200 ml of 20 mM Tris.base, pH 7.5, 200 mM NaCl, 1 mM β-ME, 1 mM EDTA, and elute it with the same buffer but 400 mM NaCl.
* Concentrate PDE4D2 with Amicon YM30 to about 10 ml.

**Sepharyl S300 column**

* Load the concentrated fractions into Sepharyl S300 column (φ2.5 x 95 cm).
* Elute the column with 20 mM Tris base pH 7.5, 1 mM β-ME, 1 mM EDTA, and 50 mM NaCl at flow rate 0.4 ml/min and 6 ml per fraction.
* PDE4D2 is eluted out at fractions 45-52.
* Concentrate it using Amicon YM30 to 8-10 units/ml.
* Store the purified protein in size of 0.5 ml at -80°C.

**\*Missing reagents and materials\***

* BL21 Competent *E.coli* cells
* Chloramphenicol
* Inhibitors **[same thing as powdered EDTA-free tablet??]**
  + PMSF
  + Leupeptin
  + Aprotinin
  + Pepstatin
* β-ME
* Ni-NTA column (φ=2.5 cm)
* 25 ml QIAGEN agarose beads
* Thrombin (Haematologic Tech. Inc)
* Q-Sepharose column (φ2.5 x 8 cm)
* Sepharyl S300 column (φ2.5 x 95 cm)
* Amicon YM30