Human PDE4D (86-413) Purification Protocol

# Plasmid amplification

## Recipe of common solution stocks

Competent Cell

(Biolab or Invitrogen) DH5-alpha or NEB5-alpha

LB medium

|  |  |  |
| --- | --- | --- |
| 1X LB 1L | | |
|  | Bacto-tryptone. | 10g |
|  | yeast extract | 5g |
|  | NaCl | 10g |
| Add H2O to 850ml | | |
| Adjust PH to 7.0 with NaOH | | |
| Add H20 to 1L | | |
| Sterilize by autoclaving | | |

Note: For plasmid amplification, you may not adjust the pH. value.

LB plate (1.5% agar) 250ml

|  |  |  |
| --- | --- | --- |
|  | 1X LB | 250ml |
|  | Agar | 3.75g |
| Sterilize by autoclaving | | |
| Wait for cooling and put in 65°C incubater | | |
|  | Ampicillin 100μg/ml | 250μl |

Or prepare in fresh (250ml)

<If you don’t like your medium to be autoclave twice>

|  |  |  |
| --- | --- | --- |
|  | Bacto-tryptone. | 2.5g |
|  | yeast extract | 1.25g |
|  | NaCl | 2.5g |
|  | Agar | 3.75g |
| Add H2O to 250ml | | |
| Sterilize by autoclaving | | |
| After temperature reducing, add ampicillin stock **375µl** | | |

Ampicillin stock 0.1g/ml

* 1g Ampicillin in 10ml
* Working concentration is 100μg/ml

80% Glycerol (50ml)

* 40ml Glycerol
* 10ml D.D. water

## Protocol

#### If you already have the cryopreservation bacteria

* Innoculate 250ml of LB with ampicillin and put 5ml of the overnight E.coli in a 1L flask. Grow for overnight (16~18hr)
* Jump to Day 3

### Day 1. Prepare the place and LB medium

* Take competent E.coli cells from –80°C freezer.
* Turn on water bath to 42°C.
* Put competent cells in a 1.5 ml tube (Eppendorf or similar). For transforming a
* DNA construct, use 50 μl of competent cells. For transforming a ligation, use 100μl of competent cells.
* Keep tubes on ice.
* Add 50 ng of circular DNA into E.coli cells. Incubate on ice for 10 min. to thaw competent cells. (15ng, 25ng, and 50ng)
* Put tube(s) with DNA and E.coli into water bath at 42°C for 45 seconds.
* Put tubes back on ice for 2 minutes to reduce damage to the E.coli cells.
* Add 1 ml of LB (with no antibiotic added). Incubate tubes for 1 hour at 37 °C.
* (Can incubate tubes for 30 minutes, unless trying to grow DNA for ligation which is more sensitive. For ligation, leave tubes for 1 hour.)
* Spread about 100 ul of the resulting culture on LB plates (with appropriate antibiotic added – usually Ampicillin or Kanamycin.) Grow 16~18hr.

### Day 2

* Pick 6~8 colonies from your selective plate into 6~8 tubes with 8ml LB with 100μg/ml Ampicillin. Each tube should only have 1 colony.
* Shake for 16~18hr at 37°c

#### Cryopreservation E. coli

* Take 3 ml bacterial cells of a freshly grown culture, put it in a 15 ml tube
* Spin for 10 minutes at 2,000 rpm
* Discard supernatant without disturbing the pellet
* Resuspend the pellet in 0.5 ml LB medium and 0.5 ml 80% glycerol
* Mix by vortexing and store in -80 °C freezer

#### If you want:

#### Sequencing

* For other 5ml bacteria, Follow the mini prep protocol to extract the plasmid for sequencing.
* For sequencing, use T7 promoter for pET15b-PDE4D2 plasmid and M13 universal reverse of forward primer for pUC19 series vector.

#### Or you can directly jump to large volume

* Innoculate 250ml of LB with ampicillin and put 2.5ml of the overnight E.coli in a 1L flask. Grow for overnight (16~18hr)

### Day 3

#### Cryopreservation E. coli

* Take 5 ml bacterial cells of a freshly grown culture, put it in a 15 ml tube
* Spin for 10 minutes at 2,000 rpm
* Discard supernatant without disturbing the pellet
* Resuspend the pellet in 0.5 ml LB medium and 0.5 ml 80% glycerol
* Mix by vortexing and store in -80 °C freezer

#### Prepare materials for Maxi prep

* Centrifuge at 6000Xg for 15min at 4°C and continue to Maxi protocol.

# BL21 Competent *E. coli* Transformation for protein expression

## Recipe of common solution stocks

Competent Cell

* BL21

40% glucose (40ml)

* 16g glucose
* 40ml D.D. water
* Sterilize by filtration (.22 filter)

Chloramphenicol Stock (20mg/ml)

* 0.2g Chloramphenicol
* Add 100% **ethanol** to 10ml
* The working concentration is 20mg per liter or 20μg/ml (1:1000 dilution)

LB plate (1.5% agar) 250ml

|  |  |  |
| --- | --- | --- |
|  | 1X LB | 250ml |
|  | Agar | 3.75g |
| Sterilize by autoclaving | | |
| Wait for cooling and put in 65°C incubater | | |
|  | Ampicillin | 250μl |
|  | Chloramphenicol | 250μl |

* Or prepare in fresh (250ml)
* <If you don’t like your medium to be autoclave twice>

|  |  |  |
| --- | --- | --- |
|  | Bacto-tryptone. | 2.5g |
|  | yeast extract | 1.25g |
|  | NaCl | 2.5g |
|  | Agar | 3.75g |
| Add H2O to 250ml | | |
| Sterilize by autoclaving | | |
|  | Ampicillin | 250μl |
|  | Chloramphenicol | 250μl |

IPTG(0.1M Stock)

* isopropyl thiogalactoside, or isopropyl beta-D-thiogalactopyranoside. Sigma stock number I5502.
* Induction of the lac operon
* I use a 0.1 M solution. The formula weight is 238.3

|  |  |
| --- | --- |
| Name | add |
| IPTG | 0.238g |
| D. D. water | 10ml |
| Sterilize by filtration, then store in the freezer. | |

## Protocol

* Thaw a tube of BL21 Competent *E. coli* cells on ice for 10 minutes.
* Prewarm the SOC medium at 42°C
* Add 1μl containing 50ng of plasmid DNA to the 100μl 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 20 seconds. Do not mix.
* Place on ice for 2 minutes. Do not mix.
* Pipette 950 μl of 42°C SOC medium 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.(Usually we do 1x, 10x, and 100x dilution)
* Spread 50–100 μl (Usually 100μl) 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

## Starting culture

For 100ml LB medium with antibiotics:

|  |  |  |
| --- | --- | --- |
| Stock | Working concentration | Add stock |
| Autoclaved LB | 1x | 100 ml |
| Ampicillin | 100μg/ml | 100 μl |
| Chloramphenicol | 20μg/ml | 100 μl |

* Transfer **one colony** of *E. coli* strain BL21 (codonplus) transformed with the vector pET15b-PDE4D (86-413) into a 10ml LB culture medium. (You will have two cryopreserved bacteria)
* Grow overnight.

If you want to stop here, follow the cryopreservation steps.

E. coli large incubation

For 500ml LB medium with antibiotics:

|  |  |  |
| --- | --- | --- |
| Stock | Working concentration | Add stock |
| Autoclaved LB | 1x | 500 ml |
| Ampicillin | 100μg/ml | 500 μl |
| Chloramphenicol | 20μg/ml | 500 μl |
| 40% Glucose | 0.4% | 500 μl |

* cryopreserve 5ml bacteria
* add other 5ml bacteria starting culture into 500ml LB
* Grow cell at 37°C to A600 = 0.7 (about 8 hours). *Use* *LB media as the reference.*

|  |  |
| --- | --- |
|  | Time |
| Start time | \_\_\_\_:\_\_\_\_ am/pm |
| 8 hours | \_\_\_\_:\_\_\_\_ am/pm |

* 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.)

## Material

CaCl2 (0.1M)

MW =

Filtered

Imidazole (150mM, 40ml)

MW = 68.08  
Imidazole: 0.408g

D.D. water: 40ml

2-Mercaptoethanol

(Density = 1.11 g/cm³, 99%, MW = 78.13, we can assume that 1ml is 1g)

0.05L \* 0.001M \* 78.13g/mole \* 1g/ml \* 1000 = 3.9 µl

The extraction buffer (50ml)

* 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
* 0.2 µg/ml pepstatin

|  |  |  |
| --- | --- | --- |
| Stock | Final Concentration | Stock add |
| 1M Tris (Filtered) | 20 mM Tris.base, pH 8.0 | 1 ml |
| 5M NaCl (Filtered) | 0.3 M NaCl | 3 ml |
| 150mM imidazole | 15 mM imidazole | 5 ml |
| β-ME | 1 mM β-ME | 3.9µl |
| PMSF | 0.2 mM PMSF |  |
| aprotinin | 0.2 µg/ml aprotinin | 10 µg |
| pepstatin | 0.2 µg/ml pepstatin | 10 µg |
| D.D. Water |  | To 50ml |

* Add 4 ml of the extraction buffer per gram of frozen cells and homogenize it.
* 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 15,000 rpm for 20 min in a JA20 rotor. Collect the supernatant.

# Ni-NTA column of His-tag PDE4D2

Buffer 1 (300ml)

|  |  |  |
| --- | --- | --- |
| Stock | Final Concentration | Stock add |
| 1M Tris (Filtered) | 20 mM Tris.base, pH 8.0 | 6 ml |
| 5M NaCl (Filtered) | 0.3 M NaCl | 18 ml |
| β-ME | 1 mM β-ME | 23.43 µl |
| 150 mM imidazole | 15 mM imidazole | 30 ml |
| D.D. Water |  | To 500ml |

Buffer 2 (200ml)

|  |  |  |
| --- | --- | --- |
| Stock | Final Concentration | Stock add |
| 1M malic acid (Filtered) | 20 mM malic acid, pH 6.5 | 4 ml |
| 5M NaCl (Filtered) | 0.3 M NaCl | 12 ml |
| β-ME | 1 mM β-ME | 15.63 µl |
| 150 mM imidazole | 15 mM imidazole | 30 ml |
| D.D. Water |  | To 500ml |

Buffer 3 (100ml)

|  |  |  |
| --- | --- | --- |
| Stock | Final Concentration | Stock add |
| 1M Tris (Filtered) | 20 mM Tris.base, pH 8.0 | 2 ml |
| 5M NaCl (Filtered) | 50 mM NaCl | 1 ml |
| β-ME | 1 mM β-ME | 7.81 µl |
| 150 mM imidazole | 15 mM imidazole | 30 ml |
| D.D. Water |  | To 500ml |

* 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 (CaCl2 stock is 0.1M, 10ml add 200µl)
  + 5µl 14 mg/ml thrombin (Haematologic Tech. Inc)
* oscillate at room temperature for digestion of one hour.
* Add 1 mM EDTA to stop the cleavage. (EDTA stock is 0.5M, 10ml add 20µl)

# Q-Sepharose column

Wash buffer (500ml)

20 mM Tris.base, pH 7.5

200 mM NaCl

1 mM β-ME

1 mM EDTA

|  |  |  |
| --- | --- | --- |
| Stock | Final Concentration | Stock add |
| 1M Tris (Filtered) | 20 mM Tris.base, pH 7.5 | 10 ml |
| 5M NaCl (Filtered) | 200 mM NaCl | 20 ml |
| β-ME | 1 mM β-ME | 39 µl |
| 0.5M EDTA | 1 mM EDTA | 1 ml |
| D.D. Water |  | To 500ml |

Elution Buffer (500ml)

|  |  |  |
| --- | --- | --- |
| Stock | Final Concentration | Stock add |
| 1M Tris (Filtered) | 20 mM Tris.base, pH 7.5 | 10 ml |
| 5M NaCl (Filtered) | 400 mM NaCl | 40 ml |
| β-ME | 1 mM β-ME | 39 µl |
| 0.5M EDTA | 1 mM EDTA | 1 ml |
| D.D. Water |  | To 500ml |

* 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

Here we refer it to wash buffer

* elute it with the elution buffer. (same buffer but 400 mM NaCl.)
* Concentrate PDE4D2 with Amicon YM30 to about 10 ml.

# Sepharyl S300 column

Elution Buffer (500ml)

|  |  |  |
| --- | --- | --- |
| Stock | Final Concentration | Stock add |
| 1M Tris (Filtered) | 20 mM Tris.base, pH 7.5 | 10 ml |
| 5M NaCl (Filtered) | 50 mM NaCl | 40 ml |
| β-ME | 1 mM β-ME | 39 µl |
| 0.5M EDTA | 1 mM EDTA | 1 ml |
| D.D. Water |  | To 500ml |

* 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.