Protocol for harvesting *pfu-sso7d* (aka Phusion) polymerase

This protocol is for expressing and purifying the high fidelity *pfu-sso7d* polymerase [1] from *E. coli*. This protein is sold as Phusion polymerase by [New England Biolabs](http://www.neb.com/%22%20%5Ct%20%22_top). This *pfu* variant has the *sso7d*processivity-enhancing domain attached that increases its speed and processivity. It generates blunt-end DNA products and typically you use higher annealing temperatures than when using *taq*.

See the [NEB website](https://www.neb.com/products/m0530-phusion-high-fidelity-dna-polymerase%22%20%5Ct%20%22_top) for a description of other key enzyme characteristics.

Expression plasmid sequence: [6his-pfu-sso7d-pET28.gbk](http://barricklab.org/twiki/pub/Lab/ProtocolsReagentsPhusion/6his-pfu-sso7d-pET28.gbk%22%20%5Ct%20%22_top)

Materials needed:

* Glycerol stock of EQ458 *E. coli* cells
The strain used is named EQ458. It is located in common species box; this is a [Rosetta 2 (DE3) E. coli strain](http://www.emdmillipore.com/life-science-research/rosetta-2de3-competent-cells/EMD_BIO-71397/p_brGb.s1OagkAAAEjQxl9.zLX%22%20%5Ct%20%22_top) containing 6his-pfu-sso7d-pET28 plasmid. The plasmid is KanR and the strain itself is CamR. The frozen stock is overnight growth of a single colony.
* LB medium. [Link to LB recipe](http://barricklab.org/twiki/bin/view/Lab/ProtocolsRecipesLuriaBertani%22%20%5Ct%20%22_top)
* Chloramphenicol stock. [Link to Cam recipe](http://barricklab.org/twiki/bin/view/Lab/ProtocolsAntibioticStockSolutions%22%20%5Ct%20%22_top)
* Kanamycin stock. [Link to Kan recipe](http://barricklab.org/twiki/bin/view/Lab/ProtocolsAntibioticStockSolutions%22%20%5Ct%20%22_top)
* Refrigerated centrifuge.
* Spectrophotometer and cuvettes.
* IPTG, 100 mM stock. Dissolve 0.238 g IPTG in 10 mL deionized water. Filter sterilize and store at –20°C.
* PMSF (1 M in ethanol)
* Disposable plastic columns. [ThermoSci, cat #29922](http://www.piercenet.com/product/disposable-plastic-columns%22%20%5Ct%20%22_top)
* Ni-NTA agarose resin. [Qiagen, cat #30210, 25 ml](http://www.qiagen.com/products/catalog/sample-technologies/protein-sample-technologies/purification-kits-and-resins/ni-nta-agarose%22%20%5Ct%20%22_top)
* Slide-A-Lyzer, 10k dialysis cassette G2. [ThermoSci, cat# 87730](http://www.piercenet.com/product/slide-a-lyzer-g2-dialysis-cassettes-10k-mwco%22%20%5Ct%20%22_top)

Lysis Buffer (for 1 purification, make 15 ml):

* 50 mM NaH2PO4
* 300 mM NaCl
* 1mM PMSF
* Adjust pH to 8.0 using NaOH

Binding Buffer (make 100 ml):

* 50 mM NaH2PO4
* 300 mM NaCl
* 10mM Imidazole
* 1mM PMSF
* Adjust pH to 8.0 using NaOH

Wash Buffer (make 50 ml):

* 50 mM NaH2PO4
* 300 mM NaCl
* 20mM Imidazole 1mM PMSF
* Adjust pH to 8.0 using NaOH

Elution Buffer (make 10-15 ml):

* 50 mM NaH2PO4
* 300 mM NaCl
* 250 mM Imidazole
* 1mM PMSF
* Adjust pH to 8.0 using NaOH

Polymerase storage buffer: Make 3-4 Liters

* 50% Glycerol
* 100 mM Tris/HCl pH 8.0
* 0.2 mM EDTA
* 0.2% NP-40; nonionic detergent
* 0.2% Tween20
* 2 mM DTT (add immediately before use)

**IMPORTANT**: Add fresh DTT immediately before use by freshly dissolving it from powder or from a 1 M stock stored at –20°C. We have observed rapid loss of function of enzyme when it is diluted in old storage buffer that has been stored at room temperature.

Protein Expression

**Day –1: Revive and Isolate Colony**

* Streak LB plate supplemented with Kan and Cam from frozen stock of EQ458. Growth plate overnight at 37°C.

**Day –2: Precondition**

* Select single colony from O/N streak plate and inoculate 1.5 mL of LB broth supplemented with Kan and Cam. Grow overnight at 37 C shaking at 250 rpm.

**Day –1: Induce**

* Use 1000 µL of overnight culture to inoculate 1 L of supplemented LB broth (in 2 L flask), grow as before for ~ 3-4 hours (5-6hrs) until an OD600 of between 0.4 and 0.6 is reached.
* Induce the cultures to express proteins by adding IPTG at a final concentration of 0.5 mM (5 mL per 1 L) followed by overnight growth at 18 C (I did this at RT, setting 7-8 on nutator), 250 rpm.

**Day 0: Harvest**

* Collect cells by centrifugation. Conditions as follows: 4°C, at 10,000 x g for 15 mins.
* Resuspend in a total volume of 12 ml lysis buffer.
* Add 1 mg/ml lysozyme and incubate rocking at 4C.
* Sonicate on setting 3-4, 45-1second pulses.
* Heat denature at 70°C for about 15 mins. Solution should become white-ish. Sit on ice for 10-15’ and then transfer to Oakridge centrifuge tube.
* Spin down in SS-34, 17,000 *rpm* (~34,000 xg) for 30 mins. Keep Supernatant!
* Proceed to IMAC purifications.

Immobilized metal ion affinity chromatography (IMAC) purification

Note: Save portions at each step for protein gel

* Prepare a 3 mL Ni-NTA resin column. The slurry is at 50%, so cut the end off a 1ml pipet tip and add 6 ml of slurry to a 50 ml conical.
* Add MilliQ water to conical, top off to 50 ml. Spin at 800xg for 1 minute.
* Gently remove water and repeat above step 3x.
* After removing water following the last step, add 10 ml binding buffer and spin.
* Repeat 3x.
* After the last spin, remove binding buffer and add your supernatant.
* Add binding buffer to reach a final volume of 40-50 ml.
* Gently rock at RT for 1-2 hrs to allow for saturated binding of Phusion to Ni-NTA resin.
* Transfer beads/supernatant to a gravity flow chromatography column.
* Allow beads to settle (5-10’) then, by gravity flow, remove the supernatant. DO NOT ALLOW BEAD BED TO DRY. Always stop the flow when the meniscus is just above the bead bed.
* Wash with 10 ml wash buffer. Repeat 2 more times.
* Elute with 6 mL of elution buffer and collect each ml fraction in separate tubes.
* Run 15-20 ul of each saved portion on an SDS-PAGE and Coomassie stain.
* *The bulk of my protein came out in fractions 3-6, with fraction #5 containing the most Phusion.*

Dialysis:

* Place dialysis cassette into storage buffer for 2 mins.
* Remove top and load dialysis cassette with enzyme sample using a pipette or syringe.
* Squeeze the membrane to remove excess air.
* Replace top and place in beaker with 1 L of storage buffer. This should be done in the cold room on a stir plate.
* Allow to sit for 2 to 4 hours.
* Remove cassette and place in beaker with fresh storage buffer. Allow to sit overnight.
* The dialysis bag will **shrink** significantly due to the dialysis solution.
* Open top of cassette and remove sample.
* Store at –20°C.

Assay purified phusion polymerase activity by PCR

\* IMPORTANT: You must use commercial Phusion buffer ([NEB Cat #B0518S](https://www.neb.com/products/b0518-phusion-hf-buffer-pack%22%20%5Ct%20%22_top)) for your reactions. It is a proprietary formulation that gives MUCH better enzyme performance.

* Template for this assay is the 6his-pfu-sso7d-pET28 plasmid encoding the phusion polymerase.
* To estimate the activity of your purified Phusion, create a dilution series of purified polymerase in water ranging from 1:200 to 1:10, and compare to NEB's stock.
* NEB stock is viscous; for an accurate comparison to the purified Phusion, ensure you are pipetting sufficient volumes to maintain accuracy.