SOP: RP029.3
Modified: 05/07/2015 PK

Production of Recombinant Proteins using a Bioreactor

Materials and Reagents:

1. BioFlo 115 7 L Fermentor (New Brunswick Scientific)
2. HyperBroth and Glucose Nutrient Solution (Athena ES Cat # AES-0107-1) (note 1)
3. LB Broth, Miller (HiVeg VWR Cat # 61000-592) (note 1)
4. LB Agar, Miller (HiVeg, VWR Cat # 61000-492) (note 1)
5. Ampicillin sodium salt, 100 mg/ml (note 1)
6. Chloramphenicol, 34 mg/ml (suspended in 200 proof ethanol)
7. Endotoxin-Free Water
8. Absolute Ethanol, 200-proof
9. Petri Dish (BD Falcon VWR Cat # 25373-100)
10. BL21 (DE3) pLysS competent *E.coli* cells and SOC media (Invitrogen # C6060-03)
11. 42^0C Water bath
12. 37^0C Shaking incubator and plate incubator
13. Ice bucket and ice
14. 500 ml flask(s), 600 ml stainless steel beaker, 500 ml bottles
15. Pipettes and tips (10, 200 and 1000 µl)
16. Luer-lok Syringes (20 and 60 ml)
17. 18G1/2 needles
18. 15 and 50 ml conical tubes
19. High-speed Centrifuge
20. 50 and 1000 ml centrifuge tubes
21. 3 L Harvesting container(s)
22. Antifoam B Emulsion (VWR # EM-R06436-74)
23. 2M Sodium Hydroxide (note 1)
24. Lysozyme (Sigma, Cat # L-6876)
25. DNase I (Sigma, Cat # D5025)
26. Complete EDTA-free protease inhibitor tablets (Roche # 40693032001)
27. Sonicator
28. Ammonium bicarbonate
29. 96-well culture plates
30. BCA protein assay kit (Pierce, Fisher # PI23227)
31. QCL-1000 Chromogenic LAL Endpoint Assay Kit (Lonza # 50-648u)
32. Optical plate reader with 405 and 550 λ filters
33. Ni-NTA Agarose His-Bind Resin (Qiagen)
34. Chromatography Column (BioRad) and stand
35. ASB-14
36. Binding Buffer (20 mM Tris-HCl, 1 M NaCl, 5 mM Imidazole, pH 7.9) (note 1)
37. Wash Buffer (20 mM Tris-HCl, 500 mM NaCl, 20 mM Imidazole, pH 7.9) (note 1)
38. 10 mM Tris-HCl, pH 8.0 (note 1)
39. Elution Buffer (10 mM Tris-HCl, 1M Imidazole, pH 8.0) (note 1)
40. 4°C Cold Room
41. Stir plate and magnetic stir bars
42. pH Meter (Tris compatible)
43. 3M KCl pH Probe Storage Solution (Mettler Toledo # C4190250)
44. 7L Dialysis Chamber
45. Dialysis tubing (Spectra-Por) or cassettes (Pierce)
46. O2-Electrolyte Solution and Reconditioning Solution (Mettler Toledo # 341002016 and 51340073)

Protocol:
Transformation (day 1):

1._____ Thaw 1 vial *E.coli* BL21 (DE3) pLysS cells and appropriate plasmid on ice (note 2).

2._____ Add 1 µl plasmid to cells and gently stir (do not pipette up and down to mix). Note lot number of plasmid in notebook

3._____ Incubate on ice 15 minutes.

4._____ Heat shock cells in 42°C water bath 30 seconds.

5._____ Recover cells on ice 2 min.

6._____ Add 500 µL SOC media to cells and shake at 37°C 1 hour.

7._____ Plate cells (50 µL) on LBAmp^{100}Cam^{34} Agar plates and incubate overnight at 37°C (usually 10 µL is enough) Spin down the remaining cells and discard the supernatant. Resuspend the pellet in 50ul fresh SOC media and plate the "Rest" of the cells on an additional LBAmp^{100}Cam^{34} Agar plate. (note 1)

Fermentor preparation and 150 ml up-scale (day 2):

Remove plate from incubator and set aside until end of day.

1._____ Rinse fermenter tank with Endotoxin-free (EF) water. Fill fermenter tank with 4.75 L EF water.

2._____ Add Hyperbroth (222.5 g) and mix (wear a mask!)

3._____ Assemble fermentor and plug in all accessories to the control tower before turning on. The probes must be autoclaved with the vessel. Make sure the protective caps are on the connection ends. The antifoam probe should be inserted all the way into the vessel. (see note 3)

4._____ Calibrate pH probe (pH 7 and 4) you may have to repeat calibration 3-4 times.

pH-calibrate-set zero-press 7-put probe in pH7 solution, wait for a stable reading-press enter-set span-press 4-put probe in pH4 solution-wait for stable reading-press enter. Calibrate pH 7 and 4 again. Test probe in pH7 solution. Repeat calibration protocol if necessary. If not necessary, assemble pH probe and dO2 probe to fermenter. Add a white gasket (top) then a black gasket (bottom) to both probes prior to assembling them to the fermenter. Set up pH probe first.

5._____ Autoclave on liquid cycle for 35 minutes (ask permission to use the large autoclave in media-prep) Note: tighten all the clamps and only keep the small liquid addition ports and the big exhaust vent open during autoclaving. Make sure all invasive and penetrating lines are closed and clamped.

6._____ Allow fermentor to cool to 55°C and plug in dO2 probe to polarize overnight.

7._____ Autoclave 2 × 150 ml LB broth, 2M NaOH, and antifoam solution. Store autoclaved NaOH and antifoam near fermenter.

8._____ Allow LB media from the two 150ml flasks to cool to 37°C and add antibiotics (Amp^{100} CAM^{34}) to the proper concentration.

9._____ Transfer 1 colony from plate to 1 of the previous media flasks and grow in shaking incubator overnight (16-18 hr) at 37°C.

Fermentation (day 3):

1._____ Set temp setting to "auto" to turn on the heat blanket (Summary-Temp, AUTO will be green). Set temperature to 37°C.

2._____ Un-plug sensor from dO2 probe to calibrate the set-zero (Calibration-DO-0-press "set zero"-0 again-reconnect). This only takes a few minutes.

3._____ Turn on gas tank (loosen the clamp on the gas tubing! A gas pressure reading of 5-8 is good, there will be danger if above 20!!, the old SOP said ≤20 is NOT correct), connect the sparge line to a 0.2 um filter and connect to the gas line of the control unit. Plug sensor back in to dO2 probe, set Gasflo to 1.0 and Agit to 200. Set agit to auto. This may take up to an hour before getting a stable reading (Temp =37°C, Agit=200, and Gas=1.0), DO concentration and saturation is a factor of temperature and thus the media must be stable at 37°C at this point during calibration. After stable reading, Summary-Air (1)-O2 ench (green), setup 100% air. Then calibrate DO to 100 (Cal-DO-set span-100).

4._____ In the meantime, remove 150 ml culture from shaker and centrifuge cells 3000 rpm/ 4°C/ 15 min.

5._____ Pour off media and use other 150 ml flask to resuspend cells in fresh LB $Amp^{100}Cam^{34}$.

6._____ Add 250 ml Glucose Nutrient Solution and 5 ml Amp^{100} and Cam^{34} to fermenter.(Use a 60 mL syringe and needle in the injection port to deliver the glucose and antibiotics to fermenter)

7._____ After DO is calibrated, set %O2 to Cascade Agit/Air (Cascade-Agit/gasflo, then back to summary); set DO setpoint to 50% (DO-setpoint-enter 50). Change gas flow from 1-5 to 0-5 and agitation limit to 200-600 in the cascade menu (Cas-Gasflo-limit).

Note: the Agit can setup from 200 to 600, do not have to setup 800 max.

8._____ Attach foam sensor to fermenter, attach the red sensor cap labeled foam/lvl 1 to the top of the antifoam probe, and then attach the clamp to the metal handle of the head plate. Now that the vessel is sterile, raise the antifoam level probe up to approximately 1-2in above the media surface. Make sure the probe insert clamp is tight, the probe should not be lowered into the media during anytime of the fermentation. Calibrate the antifoam level probe (Calibration-level 1-sensitivity). The sensitivity of the probe should be set to 90 and indicate "Dry". Take a blank media sample for spec. (OD600 mode).

9._____ Calibrate pumps: pumps-60s, start. For antifoam USE level 1, connect the clip on the handle of fermenter. Pump A: base (10% set point). Pump B: antifoam (30% set point)

10._____ Add cells to fermentor and monitor OD readings periodically (every 30-60 min). After inoculating cells, connect antifoam solution and 2M NaOH to the bioreactor pumps and the fermenter. Turn the switches to the pumps on and loosen the clip on the antifoam and NaOH tubing.

11._____ Once OD is between 2.5 and 2.9 (note: induce when the OD600 closes to 2.5, this should take approximately 4 to 5 hours), induce to final concentration of 0.5 mM IPTG (2.5 mL of 1M IPTG or 0.72g IPTG in 3 ml EF H_2O, pass through 0.20 µm filter, can get 2.5 ml 1M IPTG). Lower the %O2 to 30% and the temperature set point to 22C for overnight growth.

12._____ Attach the cooling lines to the head plate, have the inlet going from the faucet to the head plate, and the outlet going from the head plate to the drain of the sink. Turn on the water supply VERY lightly. Adjust water pressure so there is only a slight drip exiting the outlet line.

Harvest and Freeze (day 4):

1._____ Check fermentor conditions and connect harvesting container to fermentor.

2._____ Loosen the clamp on the harvesting tube and close the clamps on all other lines.

3._____ Turn off the cascade (cascade-none) and set gas to (gas-set point-2.0). This will increase the pressure inside the vessel and pump the cell culture out to the harvesting container.

4._____ Immediately after harvesting cells, turn off the gas tank and loosen the clamps on the pressure release filters. Clean the pH probe with Lysol first, then water, then store in 3M KCl storage solution. (note 4).

5._____ Pour culture into 6 × 1000 mL pre-weighed centrifuge bottles and spin down 6000 rpm/ 4°C/ 30 min.

6._____ In the meantime, turn off and disassemble the fermentor. Soak all components in disinfectant for at least 30 min before cleaning. Then wait for everything to dry completely before reassembling.

7._____ Pour supernatant back into harvesting containers and autoclave.

8._____ Weigh the bottles again to determine mass of cell pellet.

9._____ Add 5ml Binding Buffer per 1g cell pellet and mix until cells are suspended.

10._____ Round volume up to nearest 50 ml and add 1 protease inhibitor tablet per 50 ml suspension and mix. (For example, if volume of cell suspension is 225 ml, add 25 ml binding buffer to make 250 ml, then add 5 tablets).

11._____ Stir for at least one hour in cold room (or stir overnight). If you are not proceeding to "Breaking Cells" protocol now or the next day, freeze cell suspension at -80°C.

Breaking Cells and Total Protein Estimation:

1._____ Thaw cell suspension (if necessary, from -80°C to -20°C, then 4°C) and add DNase (0.05 mg/ml final) and Lysozyme (0.3 mg/ml final). Stir for at least 60 minutes at 4°C.

2._____ Transfer cell suspension to 600 ml stainless steel beaker and sonicate on ice 60 sec pulse, 90 sec rest for 10-20 cycles using large probe.

3._____ Centrifuge 16,000 ×*g* / 4°C/ 90 min to remove cellular debris and to clarify. Cell pellet may contain recombinant protein in the form of inclusion bodies. Save the cell pellet at -80°C.

4._____ Place cleared cell lysate in a 250 mL Falcon tube (note: make sure do not mix any cellular debris to the cell lysate, put the lower level of cell lysate to another centrifuge tube and Centrifuge 16,000 ×*g* / 4°C/ 90 min, if necessary). Perform BCA assay on cell lysate using 1:5, 1:20, 1:50 and 1:100 dilutions to determine total protein amount (SP003).

5._____ Run a SDS-PAGE gel and perform a Simply-blue stain on cell lysate and pellet to estimate % of recombinant protein in total protein. Estimate can also be based on past purifications (note 5).

Nickel Column:

1._____ Estimate how much nickel resin to use based on recombinant protein estimation from above. Keep in mind it is better to be conservative to avoid non-specific binding of contaminating proteins.

$$\frac{\text{x mg protein}}{\text{8 mg protein}} \times \text{1 ml packed resin} = \textbf{ml packed resin (column volume)}$$

To determine how much resin slurry to use:

$$\text{x ml packed resin} \times \frac{\text{11 ml resin slurry}}{\text{5 ml packed resin}} = \textbf{ml resin slurry}$$

Note: for CFP-10 protein, using 80%-85% of calculated volume to overload column

2._____ If using uncharged resin, refer to **Note 6** to charge the agarose resin with Nickel sulphate.

3._____ Add resin to an appropriate sized column and wash with 10 CV (column volumes) Endotoxin-free Water.

4._____ Equilibrate with 10 CV Binding Buffer.

5._____ Add equilibrated resin to cell lysate in a 200-250 mL Falcon tube. Rock overnight in cold room.

6._____ Pour cell lysate and resin back into column and collect the flow through in a 200-250 mL Falcon tube. Wash column with 20 CV Binding Buffer. Collect binding buffer fraction.

7._____ Wash with 10 CV Wash Buffer. Collect wash buffer fraction

8._____ Wash with 10 CV 10 mM Tris-HCl. Collect Tris-HCl fraction

9._____ Wash with 10 CV 0.5% ASB-14 in 10 mM Tris-HCl (must be made fresh). Collect fraction.

10._____ Wash with 10 CV 10 mM Tris-HCl. Collect fraction

11._____ Elute with 5 CV Elution Buffer (close stopcock before adding and let buffer sit in column ~ 15 minutes before opening).

12._____ Run 10 µl of each fraction on a gel and stain with Simply Blue (or Nu Blue Express). Save all column fractions until you can determine where your protein eluted off of the column, especially the flow-through, bind, and wash fractions. If a significant amount of recombinant protein is still seen in any of the fractions, you can dialyze back into Binding Buffer (if necessary) and pass back again over the regenerated column (note 6).

13._____ Dialyze elution fraction in 7L 10 mM AMBIC (pre-cooled to 4°C) in cold room. Do 2 exchanges total, at least 8 hours per exchange. For CFP10, dialyze first into 2L 10 mM Tris-HCl pH 8.0, then 2L 10 mM AMBIC, then finally into 7L 10 mM AMBIC.

Quality Control (QC) Analysis:

1._____ Carefully remove protein from dialysis and perform a BCA Assay (SP003) to determine purified protein concentration and quantity. This step must be done after dialysis has been performed, the imidazole concentration of the buffers will interfere with the BCA reaction.

2._____ Based on BCA concentration results, run on a gel 2 µg (silver stain) and 4 µg (western blot). (SP007 and SP011) (note 7).

3._____ If gel and blot look good, proceed with performing a LAL endotoxin assay. (SP020.2) (note 8).

4._____ If purified protein passes QC, fill out a QC sheet, sign it, and have it signed by a TB Contract supervisor.

5._____ Aliquot and lyophilize protein. (SP004).

6._____ Store at -80°C.

Notes:

1. Use Endotoxin-free water for all media and buffers. All buffers must also be sterile-filtered through a 0.2 µM filter. For expression of *Mycobacterium leprae* proteins, use kanamycin (50 mg/mL) and NOT ampicillin and chloramphenicol.
2. Plasmids:
    a. CFP10 pMRLB46

b. ESAT-6 pMRLB7
c. Ag85A pMRLB 41
d. Ag85B pMRLB 47

Use *E. coli* BL21 (DE3) cells for *Mycobacterium leprae* protein production

3. Refer to New Brunswick manual for detailed instructions.
4. About every 3 months or so, place the pH probe in regeneration solution overnight before storing long-term in 3M KCl.
5. % Recombinant protein in total protein (average from past batches).
   a. CFP10 1.2 % 25 mg average yield per fermentation
   b. ESAT-6 16.8 % 376 mg average yield from 1/3 cell lysate per fermentation
   c. Ag85A 2.7 % 127 mg average yield per fermentation
   d. Ag85B 1.8 % 58 mg average yield per fermentation
6. Before passing protein back over the nickel column, it must be regenerated as follows:
   a. 5 CV Strip Buffer (100 mM EDTA, 20 mM Tris-HCl, 500 mM NaCl, pH 8.0)
   b. 10 CV H20
   c. 2 CV Charge Buffer (50 mM NiSO4) let sit 10 min in column
   d. 10 CV H20
   e. 10 CV Binding Buffer
7. Antibodies to use:
   a. CFP10 α-CFP10 rabbit polyclonal or anti-penta His tag mouse monoclonal
   b. ESAT-6 α-ESAT6 rabbit polyclonal
   c. Ag85A IT-49 mouse monoclonal
   d. Ag85B IT-44 mouse monoclonal
8. Endotoxin limit for TB Contract Materials is 10 ng endotoxin per 1 mg purified protein.