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10. APPENDIX 1: MANUAL PUMP OPERATION

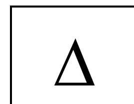
10.1. Front Panel Controls and Indicators

10.1.1 Digital Display

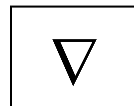
On all four pumps the 4-digit display toggles between the pump flow rate (ml/min), pressure (psi), upper pressure limit (psi), and lower pressure limit (psi). The choice of display is selected with the MODE key..

10.1.2 Keypad

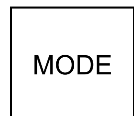
When pressed, this button alternately starts and stops the pump.



When pressed, this button increases the flow rate.



When pressed, this button decreases the flow rate.



Use this button to cycle through the four display modes: flow rate, pressure, upper pressure limit, or lower pressure limit. A status LED to the right of the digital display indicates which mode is active.

Fast And Slow Button Repeat On The Up And Down Arrow Buttons: If the UP-ARROW or DOWN-ARROW button is held down for more than approximately one half of a second, the button press will repeat at a slow rate of approximately 10 times a second. Once slow button repeat has begun, fast button repeat can be initiated by using a second finger to press down the second arrow button. During fast button repeat, the button press will repeat at a rate of approximately 100 times a second. Switching back and forth between repeat speeds can be accomplished by pressing and releasing the second arrow button while keeping the first arrow button held down.

10.1.3 Status LEDs

ML/MIN	When lit, the digital display shows flow rate in ml/min.
PSI	When lit, the digital display shows system pressure in psi.
HI PR	When lit, the display shows the user-set upper pressure limit in psi.
LO PR	When lit, the display shows the user-set lower pressure limit in psi.
RUN	Lights to indicate that the pump is running.
FAULT	Lights when a fault occurs and stops the pump.

10.1.4 Power-up Configuration

Pressure Compensation: On power-up, press the MODE button on the front panel while pressing the Power On switch on the front of the pump. The pump will display a number from 0 to 10. This represents the running pressure of the pump from 0 psi to 1000 psi. Each digit represents 100 psi. To change the pressure compensation number use the up arrow and down arrow buttons. When you have selected the correct pressure compensation press the RUN button to return to normal operation of the pump. For the Semba Octave System the value should be set to 3 (300 psi).

Non-volatile Memory Reset**:** If the pump is operating erratically, there is the possibility that the memory has been corrupted. To reset the memory and restore the pump to its default parameters, press and hold the UP-ARROW button when the power is switched on. Release the button when the display reads "rES". The parameters stored in non-volatile memory, i.e., the flow rate, the pressure compensation, the voltage/frequency select, the lower pressure limit, and the upper pressure limit will be set to the factory default values. The head type setting is the only parameter not changed by the non-volatile memory reset function. If the firmware is upgraded to a newer version, a non-volatile memory reset will automatically occur the first time the power is switched on.

10.2. Theory of Operation

10.2.1 Pump Cycle

The pump cycle consists of two phases, the pumping phase and the refill phase.

During the pumping phase, the pump piston moves at a constant linear speed, driven by a specially shaped cam which is in turn driven by the motor using a toothed-belt drive. This results in a constant, stable flow from the pump at high pressure.

At the end of the pumping phase, the pump enters the refill phase. The cam is shaped so that the piston quickly retracts, refilling the pump head with solvent. The piston then moves forward again as the pumping phase begins. Since the output flow completely stops during refill, an optional pulse damper is necessary to provide some of the lost flow. In addition, the motor speed is adjusted by the microprocessor to facilitate an efficient refill phase.

For optimal operation of the check valves, a back-pressure of at least 25 psi is required. Operating at lower pressures can result in improper seating of the valves and subsequent inaccurate flow rates.

10.2.2 Electronic Control

10.2.2.1 Microprocessor Control

The pump is controlled by hybrid microprocessor circuitry which (1) provides control signals to the motor power board, (2) interfaces with the keyboard/display, (3) receives signals from the pressure transducer and refill flag, and (4) provides external input/output and remote control interfacing. Firmware programming is stored in an EPROM.

The motor power board contains programmed logic components which (1) provide suitable motor micro-stepping modes, (2) allow appropriate motor power adjustment, (3) maximize motor power output, (4) reduce motor resonance effects, and (5) customize motor stepping uniformity. MOSFET power transistors efficiently control the motor power provided by a 36 VDC linear power supply. This board also provides the 12 VDC (linear power supply) and the 5 VDC (switching power supply) used by the pump circuits.

A specially shaped cam provides refill in a fraction of the full cam revolution. The remaining revolution of the cam provides a linear piston displacement for constant

flow of the mobile phase. In addition to the rapid refill characteristics of the cam, the onset of refill is detected by an infrared optical sensor. The microprocessor changes the refill speed of the motor to an optimum for the set flow rate. As a result, at 1 ml/min flow the refill rate is more than five times faster than if the motor operated at constant speed. The optimum refill minimizes the resulting pulsation while avoiding cavitation effects in the solvent entering the pump head.

The flow rate of any high-pressure pump can vary depending on the operating pressure and the compressibility of the fluid being pumped. The pump is calibrated at 1,000 psi using an 80:20 mixture of water and isopropanol.

10.2.2.2 DC Power Supply

Power for the pump is provided by a universal power supply which accommodates voltages of 110 to 240 VAC.

10.2.2.4 Motor Stall Detector

The motor can stall and create a loud buzzing sound if the flow path connected to the pump's outlet becomes plugged, if the pressure exceeds the maximum pressure rating of the pump, or if the mechanism jams. In the event a motor stall occurs, the electrical current being supplied to the motor is turned off and the fault light is turned on.

The Motor Stall Detector is enabled or disabled during power-up by pressing and holding the RUN/STOP and the MODE buttons when the power is switched on. Release the buttons when the display displays "SFE". To enable the Motor Stall Detector press the UP-ARROW button and the display will display "On". To disable the Motor Stall Detector press the DOWN-ARROW button and the display will display "OFF". To exit this mode and store the current setting in non-volatile memory, press the RUN/STOP button.

The Motor Stall Detector uses a timer to determine if the camshaft has stopped turning or if the refill switch is defective. The timer begins timing after the pump accelerates or decelerates to its set-point flow rate. If the Motor Stall Detector has been enabled, and the cam shaft stops turning or the refill switch stops operating, the fault will be detected between the time it takes to complete 1 to 2 pump cycles. A pump cycle is defined as the time it takes for the camshaft to complete one complete revolution. One revolution of the camshaft produces a delivery phase and a refill phase. Each specific flow rate has a corresponding cycle time. The cycle time is approximately: 30 seconds at 0.1 ml/min, 3 seconds at 1.00 ml/min, and 0.3 seconds at 10.00 ml/min.

The fault is canceled by pressing the RUN/STOP button on the front panel

11. APPENDIX 2: PUMP MAINTENANCE

Cleaning and minor repairs of the Pump Module can be performed as outlined below.

Note: Lower than normal pressure, pressure variations, or leaks in the pumping system can all indicate possible problems with the piston seal, piston, or check valves. Piston seal replacement could be necessary after 1,000 hours of running time. See Section 10.2.3.

11.1. Mobile Phase Solutions

As with any HPLC or LC system, solvent filtration is good practice for the reliability of the Quaternary Pump Module and other Octave™ system components. All solutions, including samples, should always be filtered with a 0.45 micron filter prior to use. This ensures that no particles will interfere with the reliable operation of the piston seals and check valves. Solvents in which buffers or other salts readily precipitate will need to be filtered more often. After filtration, the solvents should be stored in a closed, particulate-free bottle.

If inlet filters are used, they should be checked periodically to ensure that they are clean and not restricting flow. A restriction could cause cavitation and flow loss in the pump. Two problems that can plug an inlet filter are microbial growth and impure solvents. To prevent microbial growth, use at least 10-20% organic solvent in the mobile phase or add a growth-inhibiting compound. If you pump 100% water or an aqueous solution without any inhibitors, microbes will grow in the inlet filter over time, even if you make fresh solution every day. Always use well filtered, HPLC grade solvents for your mobile phase.

11.2. Changing Pump Heads

11.2.1 Removing the Pump Head

As a guide to pump head assembly, the standard pump heads are shown in the following diagrams. All of the Semba pump heads have a similar arrangement.

1. Turn OFF the power to the pump.
2. Remove the inlet line and filter from the mobile phase reservoir(s). Be careful not to damage the inlet filter or crimp the tubing.
3. Remove the inlet line from the inlet check valve.
4. Remove the outlet line from the outlet check valve.
5. Remove inlet and outlet self-flush lines. These may need to be cut off if removal by gentle pulling is difficult. Be careful not to damage the plastic connectors.
6. Momentarily turn ON the pump and quickly turn OFF the power upon hearing the refill stroke. This reduces the extension of the piston and decreases the possibility of piston breakage.
7. Unplug the power cord.
8. Carefully remove the two knurled nuts at the front of the pump head.

CAUTION: Be careful not to break the piston when removing the pump head. Twisting the pump head can cause the piston to break.

9. Carefully separate the pump head from the pump. Move the pump head straight out from the pump and remove it from the piston. Be careful not to break or

damage the piston. Also remove the seal and seal backup washer from the piston if they did not stay in the pump head.

10. Carefully separate the flush housing from the pump. Move the flush housing straight out from the pump and remove it from the piston. Be careful not to break or damage the piston. Also remove the self-flush seal from the piston if it did not stay in the flush housing.

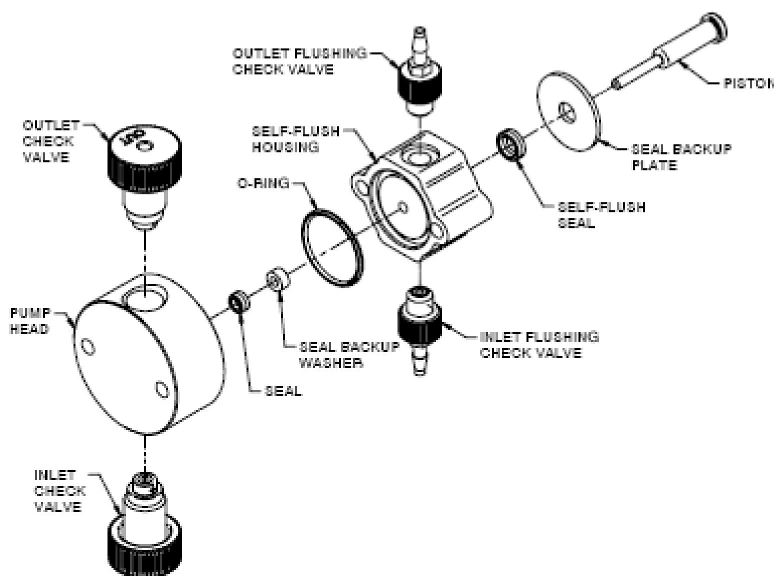


Figure 20. Self-Flushing Pump Head Assembly

11.2.2 Cleaning the Pump Head Assembly

Note: If you choose to remove the piston seal or self-flush seals, you should have a new set on hand to install after cleaning. It is not recommended that you reinstall used piston or self-flush seals since they are likely to be scratched and damaged during removal and would not provide a reliable seal if reused. If you decide to remove the seals, use only the flanged end of the plastic seal removal tool supplied with the seal replacement kit and avoid scratching the sealing surface in the pump head. See Section 11.2.3 for seal replacement instructions.

1. Inspect the piston seal cavity in the pump head. Remove any foreign material using a cotton swab, or equivalent, and avoid scratching the sealing surfaces. Repeat for the self-flush housing. Be sure no fibers from the cleaning swab remain in the components.

2. The pump head, check valves, and self-flush housing may be further cleaned using a laboratory grade detergent solution in an ultrasonic bath for at least 30 minutes, followed by rinsing for at least 10 minutes in distilled water. Be sure that all particles loosened by the above procedures have been removed from the components before re-assembly.

3. If the check valves have been removed, tighten each check valve to 10-15 inch-pounds. Be sure to insert the check valves in their proper orientation, with the arrows (embossed on the valve) pointing toward the pump head on the inlet side and away from the pump head on the outlet side (see diagram below).

4. If the piston and flushing seals have been removed, insert new seals as described in Section 11.2.3, then continue with Section 11.2.5 to replace the pump head.

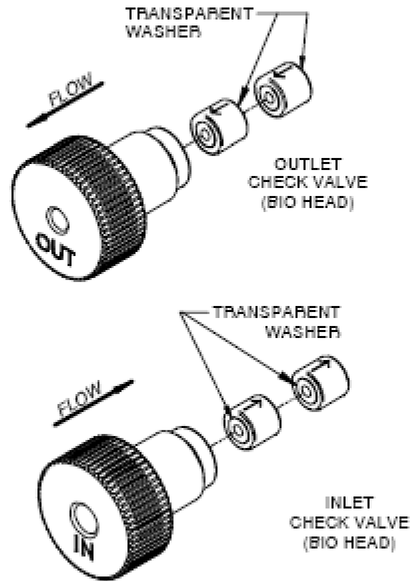


Figure 21. Check Valves

11.2.3 Replacing Piston Seals

Lower than normal pressure, pressure variations, and leaks in the pumping system can all indicate possible problems with the piston seal. Depending on the fluid or mobile phase used, piston seal replacement is often necessary after 1000 hours of running time.

Each replacement seal kit contains one seal, one backup washer, one self-flush seal, one non-flush guide bushing, two seal insertion/removal tools, and a pad to clean the piston when changing the seal.

11.2.3.1 Removing the Seals

1. Remove the pump head as described in Section 10.2.1.
2. Insert the flanged end of the seal insertion/removal tool into the seal cavity on the pump head. Tilt it slightly so that flange is under the seal and pull out the seal.

CAUTION: Using any other “tool” will scratch the finish.

3. Repeat the procedure for the low-pressure seal in the flush housing.
4. Inspect, and if necessary, clean the pump head as described in Section 11.2.2.

11.2.3.2 Cleaning the Piston

1. Once the pump head and self-flush housing are removed, gently remove the seal back-up plate by using either a toothpick or small screwdriver in the slot on top of the pump housing.
2. Grasp the metal base of the piston assembly so that you avoid exerting any side load on the sapphire rod, and remove the piston from the slot in the carrier by sliding it up.
3. Use the scouring pad included in the seal replacement kit to clean the piston. Gently squeeze the piston within a folded section of the pad and rub the pad along the length of the piston. Rotate the piston frequently to assure the entire

surface is scrubbed. Do not exert pressure perpendicular to the length of the piston, as this may cause the piston to break. After scouring, use a lint-free cloth, dampened with alcohol, to wipe the piston clean.

4. Grasp the metal base of the piston assembly, and insert it into the slot in the piston carrier until it bottoms in the slot.

11.2.3.3 Replacing the Seals

1. Place a high-pressure replacement seal on the rod-shaped end of the seal insertion/removal tool so that the spring is visible when the seal is fully seated on the tool. Insert the tool into the pump head so that the open side of the seal enters first, facing the high-pressure cavity of the pump head. Be careful to line up the seal with the cavity while inserting. Then withdraw the tool, leaving the seal in the pump head. When you look into the pump head cavity, only the polymer portion of the seal should be visible.
2. Place a self-flush replacement seal on the seal insertion/removal tool so that the spring in the seal is visible when the seal is on the tool. As in the previous step, insert the tool and seal into the seal cavity on the flushing housing, taking care to line up the seal with the cavity, and then withdraw the tool. When the seal is fully inserted only the polymer part of the seal will be visible in the seal cavity.
3. Place seal back-up washer over the high-pressure seal. Place seal back-up plate back into pump housing if it was removed. Orientation is not important in these cases.
4. Attach the pump head as described in Section 11.2.5.
5. Condition the new seal as described in Section 11.3.

11.2.4 Changing the Piston

1. Remove the pump head as described in Section 11.2.1.
2. Grasp the metal base of the piston assembly so that you avoid exerting any side load on the sapphire rod, and remove the piston from the slot in the carrier by sliding it up.
3. Grasp the metal base of the replacement piston assembly, and insert it into the slot in the piston carrier until it bottoms in the slot.
4. Attach the pump head as described in Section 11.2.5.

11.2.5 Replacing the Pump Head

1. Make sure that the inlet valve is on the bottom and the outlet valve is on the top. Carefully align the self-flush housing and gently slide it into place on the pump. If misalignment with the piston occurs, gently push up on the piston holder.
2. Line up the pump head and carefully slide it into place. Be sure that the inlet valve is on the bottom and the outlet valve is on the top. Do not force the pump head into place.
3. Finger tighten both knurled nuts into place. To tighten firmly, alternately turn nuts 1/4 turn while gently wiggling the pump head to center it.
4. Re-attach the inlet and outlet lines. Reconnect the self-flush lines and fittings to the self-flush check valves. Change the flushing solution.

11.3. Conditioning New Seals

Note: Use only organic solvents to break-in new seals. Buffer solutions and salt solutions should never be used to break-in new seals.

Using a restrictor coil or a suitable column, run the pump with a 50:50 solution of isopropanol (or methanol) and water for 30 minutes at the back pressure and flow rate listed under PHASE 1 below and according to the pump head type. Then run the pump for 15 minutes at a backpressure and flow rate listed under PHASE 2 below.

PHASE 1		PHASE 2	
Pressure	**Flow Rate**	**Pressure**	**Flow Rate**
1000 psi	< 3 ml/min	1000 psi	3-4 ml/min

11.4. Check Valve Cleaning

Many check valve problems are the result of small particles interfering with the operation of the check valve. As a result, most problems can be solved by pumping a strong solution of liquid, laboratory grade detergent through the check valves at a rate of 1 ml/min for one hour. After washing with detergent, pump distilled water through the pump for fifteen minutes. Always direct the output directly to a waste beaker during cleaning. If this does not work, the check valve should be replaced.

11.5. Cleaning the Pump

1. Disconnect the pump outlet line from the Chromatography Module and insert it into a waste beaker.
2. Set the flow rate to 10 ml/min.
3. Pump 100% isopropanol through the pump for 3 minutes.
4. Pump 100% filtered, distilled water through the pump for 3 minutes.

The pump is now prepared for any mobile phase or short- or long-term shutdown.

11.6. Cleaning the Cabinet

Cabinet may be cleaned with tap water or mild soap solution.

11.7. Lubrication

The pump has modest lubrication requirements. The bearings in the pump housing and piston carrier are permanently lubricated and require no maintenance. A small dab of a light grease such as Lubriplate 630-AA on the cam is the only recommended lubrication. Be sure not to get lubricant on the body of the piston carrier, as this can retard its movement and interfere with proper pumping.

Note: Keeping the interior of the pump free of dirt and dust will extend the pump's useful life.

11.8. Fuse Replacement

Three fuses protect the Semba pump. Two of the fuses are located in the power entry module at the rear of the cabinet and are in series with the AC input line. The other fuse is located on the motor power circuit board and is in series with the 48 VDC supply.

Troubleshooting the fuses is straightforward. If the power cord is plugged in and the ON/OFF power entry switch is ON and the fan does not run, check the two fuses in the power entry module. To gain access to these fuses, gently pry off the cover plate with a small flat-bladed screwdriver. Replace with fuses of the correct rating: 1 A slow-blo 250 VAC.

If the front panel appears to function normally but the pump motor does not run, check the fuse located on the motor power circuit board. Replace it with a 5 A slo-blo fuse.

11.9. Battery Replacement

The battery provides power for the memory that holds the current pump configuration. If the pump is set at a flow rate other than 1.00 or 10.0 and the power is turned off, when the power is turned back on the flow rate should appear, as it was set. If this flow rate does not appear the battery will need to be replaced.

1. Unplug the unit.
2. Remove the cover.
3. Turn the unit so that the control panel is to the right. The battery can be seen in the lower right corner of the circuit board. The battery is circular and has a positive pole mark (+) on the top. Gently pull it from its socket.
4. With the positive mark (+) up, gently slide the new battery into the battery socket. Be sure the battery is all the way into place. It must contact the base of the battery socket.
5. Replace the cover to the unit.
6. Plug the unit back into a properly grounded outlet.

11.10. Pump Troubleshooting

You Notice	This May Mean	Possible Cause	You Should
1. Uneven pressure trace. 2. Pressure drops. 3. Pump shuts OFF. 4. No flow out the outlet check valve.	1. Bubble in check valve. 2. Leaks in system. 3. Dirty check valve. 4. Bad check valve.	1. Solvent not properly degassed. 2. Fittings are not tight. 3. Mobile phase not properly filtered. 4. Particles from worn piston seal caught in check valve. 5. Plugged inlet filter.	1. Check to be certain that mobile phase is properly degassed. 2. Check connections for leaks by tightening fittings. 3. Prime the system directly from the outlet check valve. 4. Clean or replace the check valves. See Section 11.4. 5. Replace inlet filter.
1. Uneven pressure trace. 2. Pressure drops. 3. Fluid between the pump head and the chassis.	1. Leaks in system. 2. The piston seal(s) are worn.	1. Fittings not tight. 2. Long usage time since last seal change. 3. Salt deposits on seal (especially if buffered aqueous mobile phases are used without the self-flush head.)	1. Check all connections for leaks. 2. Replace piston seal. See Sections 11.2.3 and 11.3. 3. Check the piston for salt deposits. Clean as necessary.
Pump makes a loud clanging or slapping noise (intermittent contact with cam).	Piston carrier is catching in piston guide.	1. Cap nut screws on the pump head are loose. 2. Seal(s) are worn. 3. Piston guide is worn 4. Salt build-up on piston carrier from use of buffers. 5. Excess lubricant on piston carrier.	1. Check cap nut screws on pump head. Tighten if necessary. 2. Replace seals. 3. Replace seal backup washer and seal. See Sections 11.2 and 11.3. 4. Consider changing to a self-flushing pump head if using buffers. 5. Clean excess lubricant and dirt off piston carrier.
Pump runs for 50 pump strokes, and then shuts down.	Lower pressure limit is activating.	1. Mobile phase is not properly filtered. 2. Particles from worn seal trapped in the system (e.g., tubing, filters, injection valve, column inlet).	1. Check to be certain the low-pressure limit is set to 0 psi. 2. Only increase the low-pressure limit after the pump attains operating pressure. 3. Contact service technician.
1. Pump shuts down after run is called even with no column connected. 2. Pump runs to maximum pressure and shuts down.	Clog in fluid system.		1. Remove and clean both the inlet and bulkhead filters. 2. If the problem persists, remove tubing from system one piece at a time until you find the clogged piece. Most clogs occur outside the pump itself.
No power when pump turned ON. Fan does not run.	Blown fuses in the power entry module.	1. Power surge. 2. Internal short.	1. Replace only with the appropriate fuses 1A 250Vac. 2. Contact service technician if problem persists.
Front panel appears OK but pump motor does not run.	Blown fuse on the motor power circuit board.	1. Power surge. 2. Internal short.	1. Replace only with the appropriate fuse. 2. Contact service technician if problem persists.
PEEK fittings or components leak.	You cannot force PEEK parts with interference to seal by brute force tightening.	1. Film of fluid between surfaces. 2. Salt crystals between surfaces. 3. Scratches in mating surfaces.	1. Clean and dry mating surfaces. 2. If scratched, replace defective part.
Self-flush heads leak flush solution.	Flush area not sealed.	1. Large (Size 016) O-ring is flattened and no longer seals. 2. Head not sufficiently tightened. 3. Scratches in mating surfaces. 4. Leaky self-flush seal.	1. Replace O-ring. 2. Tighten head. 3. Replace leaky parts.

12. APPENDIX 3: PREPARATION OF PROTEIN SAMPLES FROM BACTERIAL CELL CULTURES

The scripts for step and isocratic SMBC described in Sections 7 and 8 have been developed for use with bacterial cell lysates prepared by standard protocols including physical disruption (e.g. sonication) and detergent/enzyme treatment. With either preparation method it is important to have a reasonable estimate of the total protein and target protein concentrations in the cell lysate that will be used as the Feed in the SMBC run, so that the most efficient run conditions can be established. This section provides quick protocols for cell culture, cell lysis, and sample preparation, plus data that can be used for estimating the total protein and target protein concentrations in bacterial cell lysates.

12.1. Cell culture and induction of target protein expression (pET vectors)

The protocols below give suggested procedures for cell culture and protein expression from pET vectors using either autoinduction media (Grabski et al., 2003; Studier, 2005) or IPTG induction in LB or TB medium. Depending on the recombinant strain, cell density measured by optical density at 600 nm can be up to 20 with wet cell paste yields up to 25 g/L.

12.1.1 Autoinduction shake-flask protocol

1. Streak the recombinant strain for single colonies on LB agar plates containing appropriate antibiotics and incubate overnight at 37°C.
2. For each liter of eventual culture, inoculate duplicate 3-ml cultures of (pre-warmed) LB or TB + antibiotics + 0.25% glucose with a single colony/culture.
3. Incubate with shaking for 1.5-2 h at 300 rpm at 37°C. Measure OD_{600} (should read 0.5 to 1.0), and add an entire 3-ml culture to each of two 2.7-L Fernbach flasks containing 500 ml pre-warmed autoinduction medium + antibiotics + 0.5% glucose. Reducing the induction culture temperature can increase soluble expression levels for some proteins. Typically 23°C is a good starting point for proteins of unknown soluble expression level.
4. Continue incubation at the induction temperature for 16-18 h. Monitor cell growth by reading the OD_{600} of a 1:20 dilution in medium at several intervals. Harvest the culture when stationary phase is reached. Optimal harvest time is strain- and temperature-dependent. It is important to incubate the culture into stationary phase to achieve maximal protein expression. Harvesting several hours after stationary phase is reached usually has no deleterious effects.
5. Divide the culture into tared centrifuge bottles or tubes and harvest cells by centrifugation 9,000 x g for 15 min. Carefully remove the supernatant and drain the pellets by inverting the bottles for a few minutes on paper towels. Record total pellet weight. Pellets can be stored at –70°C until they can be further processed.

12.1.2 IPTG induction shake-flask protocol

1. Streak the recombinant strain for single colonies on LB agar plates containing appropriate antibiotics and incubate overnight at 37°C.
2. For each liter of eventual culture, inoculate duplicate 3-ml cultures of (pre-warmed) LB or TB + antibiotics + 0.5% glucose with a single colony/culture.
3. Incubate with shaking for 1.5-2 h at 300 rpm at 37°C. Measure OD_{600} (should read 0.5 to 1.0), and add an entire 3-ml culture to each of two flasks containing 50 ml prewarmed LB or TB + antibiotics + 0.5% glucose.

4. Incubate with shaking for 1.5-2 h at 300 rpm at 37°C. Measure the OD_{600} (should read 0.5 to 1.0), and add an entire 50-ml culture to each of two 2.7-L Fernbach flasks containing 500 ml pre-warmed LB or TB medium + antibiotics + 0.5% glucose.
5. Incubate with shaking at 37ºC, 300 rpm until OD_{600} reaches 1.0 to 1.5. If reduced temperature induction is desired, the incubator temperature should be decreased when the OD_{600} reaches ~0.9 to allow for culture cooling prior to IPTG addition.
6. The inducer IPTG should be added when the culture reaches an OD_{600} of approximately 1-1.5. Add IPTG from a filter-sterilized 1 M stock (typically 1 mM final conc.).
7. Continue incubation with shaking at the desired induction temperature and harvest cells when peak expression is achieved (e.g. 2-4 h at 37°C).
8. Divide the culture into tared centrifuge bottles or tubes and harvest cells by centrifugation 9,000 x g for 15 min. Carefully remove the supernatant and drain the pellets by inverting the bottles for a few minutes on paper towels. Record total pellet weight. Pellets can be stored at –70°C until they can be further processed.

12.2. Bacterial cell lysis and preparation of the soluble fraction (Feed)

It is highly recommended to prepare cell lysates immediately before performing chromatography to maximize protein activity and avoid the formation of precipitates that can interfere with operation of the Octave™ instrument. Do not freeze cell lysates, because freezing and thawing can cause precipitation and loss of protein activity.

12.2.1 Cell lysis by detergent and enzyme treatment

A convenient method for preparing protein samples from bacterial cells is to use a combination of detergents and enzymes. Several commercial products are available for this purpose. A non-ionic detergent is used which does not interfere with enzyme activity or protein function in the vast majority of cases. Enzymes include lysozyme, which aids in breakdown of the bacterial cell wall, and Benzonase®, which degrades all forms of DNA and RNA, reducing viscosity and interference from nucleic acids in the lysate.

1. Completely resuspend the cell pellet in 5 ml detergent/enzyme mixture per g cell pellet. Additives such as protease inhibitors, 5-10% glycerol, and reducing agent can be included in the buffer to limit proteolysis and stabilize labile proteins. These additives must be compatible with the chromatographic separation method chosen for purification. It is very important to mix thoroughly until the solution is homogeneous and free of clumps or debris. Mix at low speed to avoid excessive foaming of the mixture and allow to react 15-20 min at ambient temperature.
2. If the lysate will be used for IMAC, add 5 ml/g cells of ice-cold 50 mM sodium phosphate, pH 8, 300 mM NaCl. Other compatible buffers (5 ml) may be substituted for other chromatography methods. Generally cell lysates should be processed as quickly as possible and kept on ice or at 4°C to minimize proteolysis. If desired, save a 50 µl sample of the lysate at this point (prior to centrifugation) for analysis of total cellular protein.
3. Centrifuge the lysate at 9,000 x g for 15 min at 4°C. Carefully transfer the supernatant (containing the soluble proteins) to another container without disturbing the pellet. Using low-protein binding filters (e.g. Corning SFCA), filter the supernatant twice, first through a 0.8 µM filter, then through a 0.45 µM filter. If using syringe filters the two filters can be attached in tandem to a syringe with

the 0.45 μM filter on the bottom. This final clarified supernatant represents the soluble cell protein fraction and is ready for chromatography.

12.2.2 Cell lysis by physical disruption

Various methods are available for preparing bacterial cell lysates using physical disruption, including sonication, French Press, and Microfluidizer. The following protocol describes the use of sonication. For disruption using French Press or Microfluidizer refer the manufacturer's instructions.

1. Completely resuspend the cell pellet by vortexing or homogenization in 10 ml/g cells 50 mM sodium phosphate buffer pH 8 containing 300 mM NaCl (for IMAC purification). Other compatible buffers (10 ml) may be substituted for other chromatography methods. Benzonase and/or lysozyme may be added to facilitate lysis and degradation of nucleic acids. Additives such as protease inhibitors, 5-10% glycerol, and reducing agent can be included in the buffer to limit proteolysis and stabilize labile proteins. These additives must be compatible with the chromatographic separation method chosen for purification. Mix at low speed to avoid excessive foaming of the mixture.
2. Sonicate the suspension on ice using 30-60-second bursts. Avoid excessive heating and aeration by adjustment of instrument settings and duration. For 50 ml BL21 cell suspension, 3 bursts at a setting of 8 and 70% duty cycle using a Branson Model 450 sonifier is usually sufficient. If desired, save a 50 μl sample of the lysate at this point (prior to centrifugation) for analysis of total cellular protein.
3. Centrifuge the lysate at 9,000 x g for 15 min at 4°C. Carefully transfer the supernatant (containing the soluble proteins) to another container without disturbing the pellet. Using low-protein binding filters (e.g. Corning SFCA), filter the supernatant twice, first through a 0.8 μM filter, then through a 0.45 μM filter (e.g. Corning). If using syringe filters, the two filters can be attached in tandem to a syringe with the 0.45 μM filter on the bottom. This final clarified supernatant represents the soluble cell protein fraction and is ready for chromatography.

12.3. Estimation of total and target protein concentrations

Total protein concentration in crude lysates can be determined by the bicinchoninic acid (BCA) method (Smith et al., 1985) or other protein assays. Target protein expression levels can be determined using target protein-specific assays and/or immunoassays, if reagents are available. Purified protein concentrations can be determined by the BCA method or spectrophotometrically at 280nm (Grimsley and Pace, 2004; Gill and von Hippel, 1989].

A convenient method to quickly estimate the target protein expression level is by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of the cell lysate followed by Coomassie blue staining. By comparing band intensities vs. known standards the target protein level can be estimated fairly accurately. Semba's Express-timator™ Kit provides a set of standards consisting of a soluble BL21 lysate containing known amounts of a 52.2-kDa protein.

12.3.1 Express-timator™ Kit protocol

1. Prepare the soluble cell lysate as described in Section 12.2.
2. Mix 50 μl lysate with 50 μl 2X SDS-PAGE sample buffer (Grabski and Burgess, 2002).
3. Immediately heat the sample at 85°C for 3 min.
4. Centrifuge at 12-17,000 x g for 2 min.

5. Load 10 µl/lane on an appropriate SDS-polyacrylamide gel (e.g. 18-well Bio-Rad Criterion™ or 10-well Invitrogen Novex® gel).
6. Load 10 µl/lane Express-timator standards in adjacent lanes.
7. Run the gel according to the manufacturer's instructions, stain with Coomassie blue, destain, and compare the expressed target band to the Express-timator™ standards to estimate the target protein expression level.

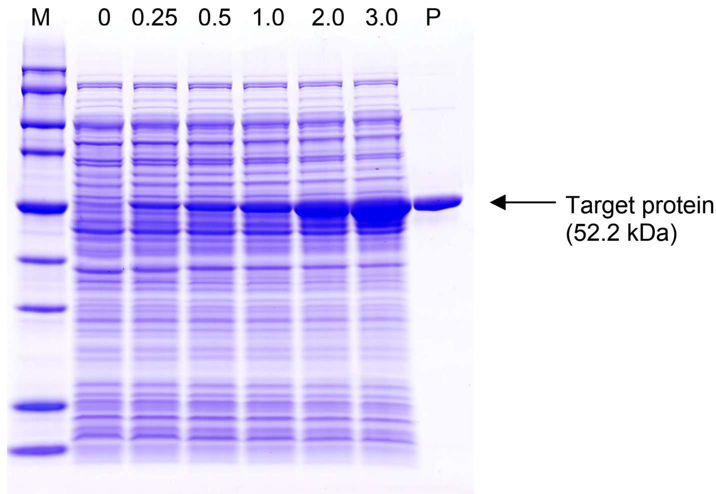


Figure 22. SDS-polyacrylamide gel analysis of Express-timator™ standards

Samples (10 µl) of the Express-timator™ Kit standards were run on a Bio-Rad Criterion™ 10-20% gradient gel and stained with Coomassie blue. Size markers (M; Novagen Perfect Protein™ 10-225 kDa) and the purified 52.2 kDa target protein (P) present in the standards were run in adjacent lanes. Numbers above the Express-timator standards correspond to mg/ml target protein.

12.3.2 Estimation of target protein concentration

Cell lysates prepared as above using BL21-derived *E. coli* host strains can be expected to contain 10.5-12.5 mg/ml total protein, 8-10 mg/ml soluble protein, and up to 5.5 mg/ml recombinant target protein, depending on the expression efficiency.

The Express-timator standards correspond to soluble cell lysate at a total protein concentration of 10 mg/ml, including the target protein. The target protein concentrations in the standards and their percentages of the total protein are given in the following table.

Express-timator™ Standard	52.2-kDa Target Protein Concentration	% Total Protein
0	0 (negative control)	0%
0.25	0.25 mg/ml	2.5%
0.5	0.5 mg/ml	5.0%
1	1.0 mg/ml	10%
2	2.0 mg/ml	20%
3	3.0 mg/ml	30%

Due to the 1:1 dilution with SDS sample buffer the actual protein concentration in the Express-timator standards is 5.0 mg/ml, such that a 10-µl sample represents 50 µg total protein.

Compare the intensity of the target protein band in your sample vs. the Express-timator standards to determine the one that most closely matches a known concentration. Use the corresponding script in Section 8 for purification with the Octave instrument.