SOP: Buck Scientific BLC-30G HPLC Operation

Approvals
Preparer: John Buford, Jason McMillan, Jack O'Neill   Date: 16JUL15
Reviewer: Dr. Maggie Bryans   Date: 17JUL15

1. **Purpose**
   1.1. Basic operation of the Buck Scientific BLC-30G gradient HPLC system in order to assay a sample using reverse phase high performance liquid chromatography (RP-HPLC).
2. **Scope and Applicability**
   2.1. High performance liquid chromatography (HPLC) is an analytical chemistry technique for separating the components of a liquid sample and for identifying and quantifying the components of the sample. This SOP provides the basic operations required to perform an assay using the Buck Scientific BLC-30G gradient HPLC system, a reverse phase HPLC column, and a compatible mobile phase solution. Other process-specific SOPs are intended to provide the details of HPLC column selection, mobile phase solution preparation, sample preparation, flow rates, and run times.
3. **Summary of Method**
   3.1. Prepare the mobile phase and storage solutions
   3.2. Power up the HPLC system components and start the PeakSimple data collection software
   3.3. Equilibrate the system with mobile phase solution
   3.4. For each sample, run an assay:
      3.4.1. Use PeakSimple to start a new run
      3.4.2. Load and inject the sample
      3.4.3. Collecting data and store data
      3.4.4. Re-equilibrate the system if directed by the process-specific SOP
   3.5. Wash the system with storage solution
   3.6. Power down the system
4. **References**
   4.1. SOP: Degassing a Solution by Helium Sparge
   4.2. BLC-30G Easy Installation and Quick Start Guide
5. **Definitions**

CV	Column Volume; the volume (mL) of the column containing the stationary phase; CV=2.91 mL for a standard size (4.6 X 250 mm) column
Equilibration	Running the mobile phase solution through the column prior to injecting the sample in order to bring the system into equilibrium
Flow rate	The rate (mL/min) that solution is pumped through the column. The operating flow rate is determined by the assay protocol, generally 1.0 mL/min for a standard size (4.6 X 250 mm) column
Helium sparge	Using a stream of helium bubbles to sweep dissolved air out of liquids (helium is virtually insoluble in most HPLC solvent solutions, so very little helium replaces the air)
HPLC	High Performance Liquid Chromatography

SOP: Buck Scientific BLC-30G HPLC Operation

Isocratic	The composition of the mobile phase solution is constant; the system has only one pump.
Mobile phase	The solvent solution used to carry the sample through the column
PeakSimple	Software used to collect and display data
PSI	Pounds per Square Inch
Reverse phase chromatography	Separation based on hydrophobicity under conditions where the stationary phase is more hydrophobic than the mobile phase.
Stationary phase	The chromatography matrix through which the sample travels.

6. **Precautions**
   6.1. Most solvents used for HPLC are toxic and flammable. For each solvent, read the Material Safety Data Sheet (MSDS) for hazards, handling and storage information. Wear personal protection equipment (PPE) and use a fume hood as required. Store solvents as indicated by the MSDSs.
   6.2. HPLC systems operate at high pressures. Personnel injury and equipment damage can result if maximum pressure is exceeded or the pump runs dry. Monitor pressure readings and solution level whenever the pump is running. If pressure exceeds 2500 psi or if the solution runs out, stop the pump immediately by pressing the RUN/STOP button. Do not set the flow rate higher than 1.5 ml/min with a 250 mm column.
   6.3. Different mobile phase solutions interact with the stationary phase differently, resulting in different back pressures for a given flow rate (see Table 3 for example pressure readings for mobile phase solutions). When changing mobile phase solutions, monitor pressure readings carefully while running the first 5 CV of the new solution as it replaces the old solution in the lines and column.
   6.4. Flow rate consistency is affected by the quality of the solutions. Use HPLC-grade solvents and filter solutions using a sub-micron filter (preferably 0.22 μm). Degas solutions prior to use.
   6.5. To avoid microbial growth, do not leave the system in a high aqueous solution for a prolonged period. The system should be washed with a storage solution of 50% Methanol/H_2O or 50% Acetonitrile/H_2O if it is to be idle more than a few hours.

7. **Responsibilities**
   7.1. It is the responsibility of the course instructor/lab assistant to ensure that this SOP is performed as described and to update the procedure when necessary.
   7.2. It is the responsibility of the students/technician to follow the SOP as described and to inform the instructor about any deviations or problems that may occur while performing the procedure.

8. **Equipment and Materials**
   8.1. Buck Scientific BLC-30G HPLC system pre-configured with:
      8.1.1. UV-Vis detector
      8.1.2. BLF-10 Fluorescence Detector
      8.1.3. PeakSimple Chromatography Data System
      8.1.4. Computer system with PeakSimple software installed
      8.1.5. Reverse phase HLPC column
   8.2. HPLC-grade solvent for mobile phase solution

SOP: Buck Scientific BLC-30G HPLC Operation

8.3. HPLC-grade methanol for storage solution

8.4. HPLC-grade water

8.5. Chemically compatible sub-micron filters (preferably 0.22 μm)

8.6. 2 laboratory bottles for mobile phase solution and waste

8.7. 2 laboratory bottles for storage solution and waste

8.8. Small bottle for mobile phase (to be used for cleaning the sample syringe)

8.9. Sample overflow waste beaker

8.10. 25 mL Luer-Lok syringe

8.11. 100 μL HPLC sample syringe

8.12. Parafilm

8.13. Timer

9. **Procedure**

Note: this BLC-30G Gradient HPLC is configured with two pumps, a UV-Vis detector, an add-on fluorescence detector, and a four channel serial port connected to a computer running PeakSimple software. The pumps and detectors are either controlled by the HPLC front panel (Figure 2) or the PeakSimple software, with the Run/Stop functions accessed through the Relay and Pump window. Data are collected and displayed by the PeakSimple software on the computer. For example, the UV-Vis data and Gradient functions are monitored/set through Channel 1. The FL data and Pump B functions are monitored/set through Channel 2. The Pressure is monitored through Channel 6.

9.1. ***Prepare mobile phase and storage solutions***:

9.1.1.   A process-specific SOP should provide the composition and volume of mobile phase required. For an example, see Table 2. Example Solution Volume Calculations.

9.1.2.   Prepare the mobile phase solution into a labeled laboratory bottle that is sized appropriately for degassing per the Degassing a Solution by Helium Sparge SOP. Filter the solution using a sub-micron filter (preferably 0.22 μm) that is chemically compatible.

9.1.3.   Prepare a minimum of 300 mL 50% Methanol/H_2O solution into a labeled 500 mL laboratory bottle. Filter the solution using a sub-micron filter (preferably 0.22 μm).

9.1.4.   Degas the mobile phase & storage solutions per the Degassing a Solution by Helium Sparge SOP.

9.1.5.   Transfer approximately 10 mL of mobile phase solution to a small labeled bottle to be used for rinsing the sample syringe.

9.1.6.   Label an empty laboratory bottle as storage solution waste. Label another empty bottle as mobile phase solution waste.

9.2. ***Power up the HPLC system components and start the PeakSimple data collection software:***

9.2.1.   Power up the computer system and monitor, then login.

9.2.2.   Power up the pump units UV-Vis detector, and fluorescence detector using the red switch located on the power strip to the left of the unit.

9.3. **Launch the PeakSimple data collection software:**

SOP: Buck Scientific BLC-30G HPLC Operation

9.3.1.1. Double click on the PeakSimple icon on the desktop. (Alternatively, navigate to C:\ Peak426-32bit and run Peak426-32bit.exe.) The PeakSimple window should appear as the software automatically connects to the HPLC hardware.

9.3.1.2. Navigate to "Edit" in the top left corner and select "Channels" from the dropdown menu.

9.3.1.3. Next to Channel 1:Uv-Vis, ensure that "active," "display" and "integrate" all have checks in them.

9.3.1.4. Select "Details"

9.3.1.5. Under the section "Control by" select "Gradient"

9.3.1.6. Under "End Time" input a value in minutes for the length of the run.

9.3.1.7. Press "OK" when finished.

9.3.1.8. Select "Gradient"

9.3.1.9. Select "Clear"

9.3.1.10. Select "Add"

9.3.1.11. Fill in the values to create the desired gradient.

9.3.1.12. If multiple gradient steps are necessary, repeat steps 9.2.3.9. and 9.2.3.10.

9.3.1.13. Press "OK" when finished.

9.3.1.14. Next to Channel 2: Fluorescence, ensure that "active," "display" and "integrate" all have checks in them.

9.3.1.15. Select "Details"

9.3.1.16. Under the section "Control by" select "Gradient"

9.3.1.17. Under "End Time" input a value in minutes for the length of the run.

9.3.1.18. Press "OK" when finished.

9.3.1.19. Next to Channel 6, ensure that "active," and "display" have checks in them.

9.3.1.20. Select "Details"

9.3.1.21. Ensure that "Datalogger mode" has a check in the box next to it.

9.3.1.22. Ensure Offset = 0

9.3.1.23. Ensure Gain = 1

9.3.1.24. Ensure Decimal Places = 0

9.3.1.25. Under "End Time" input a value in minutes for the length of the run.

9.3.1.26. Press "OK" when finished.

9.3.1.27. Press "OK" to close the Channels window.

9.3.1.28. Allow the detectors to warm up for 60 minutes prior to collecting data.

9.4. *Switch the system to mobile phase solution:*

9.4.1. Verify that the pump is off. The Run LED should be off.

9.4.2. Place the intake lines into the mobile phase solution bottle and cover with Parafilm. Verify that the frit is submerged in the solution.

9.4.3. Place the outlet line into the mobile phase waste bottle.

SOP: Buck Scientific BLC-30G HPLC Operation

9.4.4.   Place the sample overflow line into a small waste beaker.

*9.5. **Purge the intake line and prime the pump:***

9.5.1.   Attach an empty 25 mL Luer-Lok syringe to the purge valve (Figure 3).

9.5.2.   Open the prime/purge valve by turning it two full turns counter-clockwise.

9.5.3.   Watching the intake line for bubbles, slowly draw the syringe plunger until it is fully drawn. Mobile phase solution and bubbles should fill the syringe.

9.5.4.   Close the prime/purge value by rotating it clockwise until it stops. Stop the pumps.

9.5.5.   Remove the syringe from the purge valve and expel the contents into the waste bottle.

9.5.6.   Repeat attaching the syringe to the purge valve, drawing bubbles and solution into the syringe, and expelling into the waste bottle until free of bubbles (generally 20-to-30 mL of mobile phase are needed).

*9.6. **Start the pump and gradually increase the flow rate to the operating rate:***

9.6.1.   A process-specific SOP should provide the flow rate for the mobile phase.

9.6.2.   Set the initial flow rate to 0.1 mL/min:

9.6.2.1. Press the MODE button on the front panel repeatedly until the Flow LED turns on. The current flow rate (in ml/min) appears on the digital display.

9.6.2.2. Press the Flow up arrow button to increase the flow rate setting and press the down arrow button to decrease the flow rate setting.

9.6.2.3. Repeat pressing the Flow arrow buttons until 0.10 is displayed.

9.6.3.   Start the pump by pressing the RUN/STOP button. The Run LED should turn on.

9.6.4.   Monitor the pressure readings and solution level:

9.6.4.1. Display the pressure reading by pressing the MODE button repeatedly until the Pressure LED turns on. The current pressure (in psi) appears on the digital display.

9.6.4.2. Verify that that solution is dripping into the waste bottle.

9.6.4.3. If pressure exceeds 2500 psi or if the solution runs out, stop the pump immediately by pressing the RUN/STOP button.

9.6.5.   Gradually increase the flow rate in 0.1 mL/min increments over a period of 5 minutes to the specified flow rate:

9.6.5.1. Press the MODE button on the front panel repeatedly until the Flow LED turns on. The current flow rate (in ml/min) appears on the digital display.

9.6.5.2. Increase the flow rate setting by 0.1 mL/min by pressing the Flow up arrow button.

9.6.5.3. Monitor the pressure readings and solution level.

9.6.5.4. Repeat increasing the flow rate setting by 0.1 mL/min increments over a period of 5 minutes until the specified flow rate is achieved and pressure readings stabilize.

*9.7. **Set the UV-Vis detector wavelength and autozero the detector:***

9.7.1.   A process-specific SOP should provide the assay run time and the UV-VIS detector wavelength.

9.7.2.   Set the UV-Vis detector wavelength by pressing the λ up and down buttons on the front panel until the specified wavelength is displayed.

SOP: Buck Scientific BLC-30G HPLC Operation

9.7.3.   Autozero the UV-Vis detector by pressing the AUTOZERO button on the front panel.

9.8. ***Equilibrate the system by running mobile phase:***

9.8.1.   A process-specific SOP should provide the equilibration run time. The run time may be expressed in terms of column volumes (CV); see Equation 1 for an example of converting a CV to a run time.

9.8.2.   Operate the pump to run for the specified run time. Monitor the pressure readings and solution level. Monitor detector values and notify the instructor if the values appear to be unstable.

9.8.3.   Alternatively, run an assay of a blank (see below) using mobile phase solution as the sample.

9.9. ***For each sample, run an assay:***

9.9.1.   Use PeakSimple to start a new run and edit the run time:

9.9.1.1. Select File > New from the menu bar.

9.9.1.2. Select Edit > Channels… from the menu bar. The Channels dialog box should appear.

9.9.1.3. Select Channel 1: Details from the Channels dialog box. The Channel details dialog box should appear.

9.9.1.4. Ensure previously set values are still in place.

9.9.1.5. Verify that the "Remote start" check box is checked.

9.9.1.6. Press "OK" when finished.

9.9.1.7. Select Channel 2: Details from the Channels dialog box. The Channel details dialog box should appear.

9.9.1.8. Ensure previously set values are still in place.

9.9.1.9. Verify that the "Remote start" check box is checked.

9.9.1.10.   Press "OK" when finished.

9.9.1.11.   Select Channel 6: Details from the Channels dialog box. The Channel details dialog box should appear.

9.9.1.12.   Ensure previously set values are still in place.

9.9.1.13.   Verify that the "Remote start" check box is checked.

9.9.1.14.   Press "OK" when finished.

9.9.1.15.   Press "OK" to close the Channels window.

9.9.2.   Autozero the UV-Vis detector by pressing the AUTOZERO button on the front panel.

9.9.3.   Load and inject the sample:

9.9.3.1. Verify that the injector port handle is set to the "Load" position.

9.9.3.2. Fill the HPLC sample syringe with 100 µL of sample, using care to avoid bubbles in the syringe.

9.9.3.3. Insert the syringe needle into the sample injection port (Figure 4).

9.9.3.4. Depress the syringe plunger, using care to avoid introducing bubbles. (Often there is a small bubble at the base of the plunger. Watch carefully and stop depressing the plunger before the bubble is loaded into the injector port. It is OK to leave a few µL of sample in the syringe.)

SOP: Buck Scientific BLC-30G HPLC Operation

    9.9.3.5. Turn the injector port handle clockwise from the "Load" to the "Inject" position (Figure 5). Note, the PeakSimple software has started displaying the elapsed run time in upper right corner.

    9.9.3.6. After 10 seconds, turn the injector port handle counter-clockwise from the "Inject" back to the "Load" position.

    9.9.3.7. Remove the syringe from the sample injection port.

    9.9.3.8. Rinse the syringe by filling it from the small bottle of mobile phase solution and expelling it into the mobile phase waste bottle at least three times.

  9.9.4. Operate the pump for the specified run time. Monitor pressure readings and solution level. At the end of the run time, note that PeakSimple elapsed run time switches to STANDBY in upper right corner.

  9.9.5. Use PeakSimple to view results and save the data to a chromatogram file:

    9.9.5.1. Select View > Results… from the menu bar. The Results dialog box should appear.

    9.9.5.2. Click the Copy button to copy the data.

    9.9.5.3. Paste the data into an Excel spreadsheet.

    9.9.5.4. Close the Results dialog box.

    9.9.5.5. Select File > Save as… from the menu bar. The Save as dialog box should appear.

    9.9.5.6. Enter a directory and a meaningful file name (e.g. operator initials, experiment name, and run number). Click the Save button.

  9.9.6. Re-equilibrate the system if directed by the process-specific SOP.

  9.9.7. Repeat this section for each sample.

9.10.   ***Stop the pump:***

  9.10.1. Press the RUN/STOP button. The Run LED should turn off and pressure readings should decrease gradually.

  9.10.2. Monitor the pressure until it decreases to less than 100 psi.

9.11.   ***Wash the system by running 5 CV of storage solution:***

  9.11.1. Switch the system to storage solution per the instructions in section 9.4 above.

  9.11.2. Purge the intake line and prime the pump per the instructions in section 9.5 above.

  9.11.3. Set the initial flow rate to 0.1 mL/min.

  9.11.4. Start the pump and gradually increase the flow rate to 0.5 mL/min over a period of 5 minutes.

  9.11.5. Operate the pump for 30 minutes. Monitor pressure readings and solution level.

  9.11.6. Stop the pump and allow the pressure to decrease to less than 100 psi.

9.12.   ***Power down the system:***

  9.12.1. Exit PeakSimple by selecting File > Exit from the menu bar. A prompt should appear asking Save all before exiting? Click the No button.

  9.12.2. Power down the pump units and the detectors using the red switch located on the power strip to the left of the pumps.

  9.12.3. Power down the Computer system.

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Document Number: QCB 9
Revision Number: 0
Effective Date: 17JUL15
Page 8 of 12

SOP: Buck Scientific BLC-30G HPLC Operation

10. Attachments

Figure 1. Buck Scientific BLC-20G HPLC System with Pumps and Detectors

SOP: Buck Scientific BLC-30G HPLC Operation

Figure 2. HPLC Front Panel
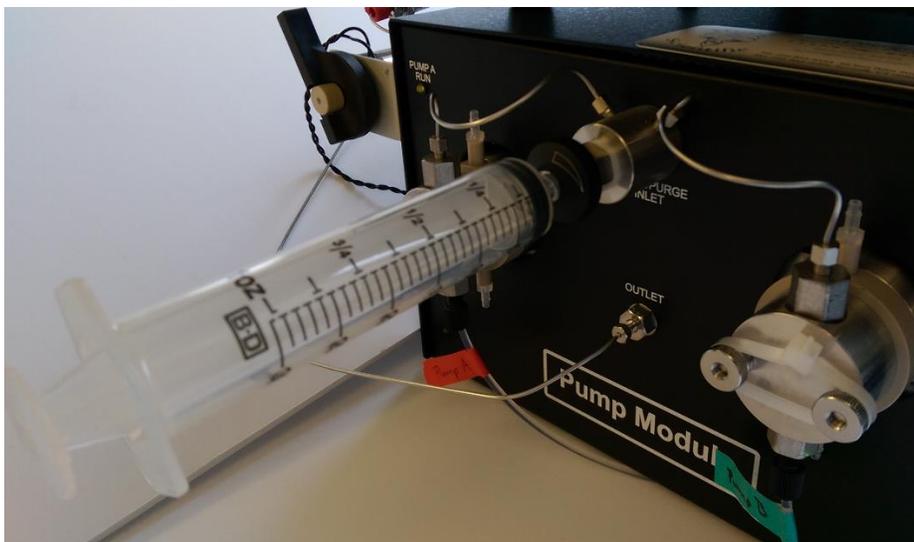
Figure 3. Purge Valve with 25 mL Luer-Lok Syringe Attached

SOP: Buck Scientific BLC-30G HPLC Operation
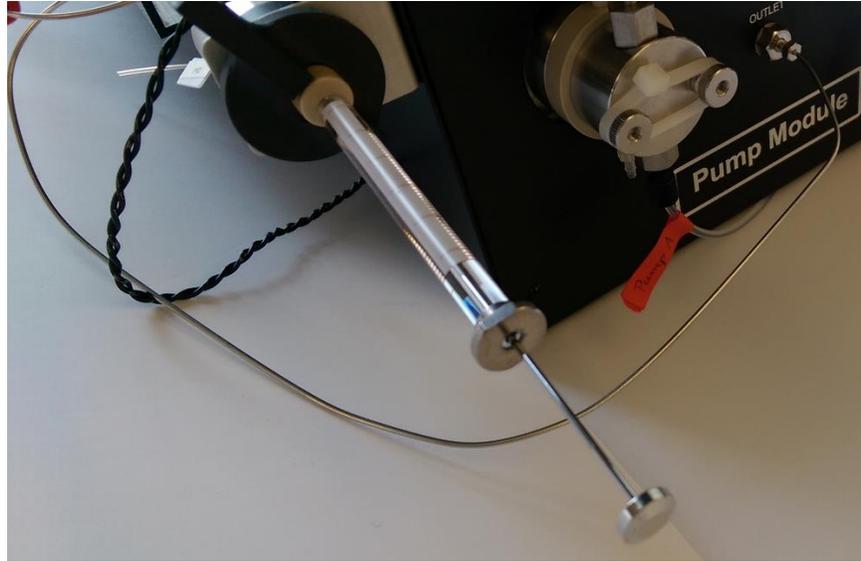
Figure 4. Sample Injection Port with HLPC Syringe Attached in 'Load' Position
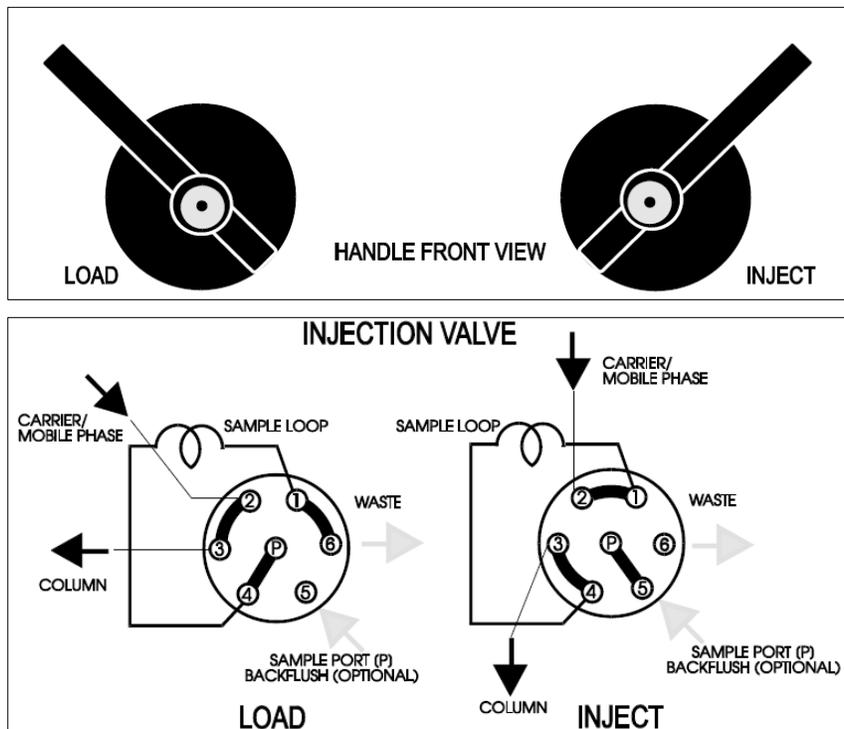
Figures 5. Sample Injection Port Positions: 'Load' and 'Inject'

Montgomery County Community College
340 DeKalb Pike
Blue Bell, PA

Document Number: QCB 9
Revision Number: 0
Effective Date: 17JUL15
Page 11 of 12

SOP: Buck Scientific BLC-30G HPLC Operation

Table 1. Common Column Volumes

Column dimension	Column volume (mL)
250 x 4.6 mm	2.91
150 x 4.6 mm	1.74
100 x 4.6 mm	1.16
50 x 4.6 mm	0.58
250 x 4.0 mm	2.20
125 x 4.0 mm	1.10
250 x 2.0 mm	0.55
150 x 2.0 mm	0.33
50 x 2.0 mm	0.11

$$CV = \pi r^2 L$$
where:

CV = column volume in mL
r = column radius in cm
L = column length in cm

Table 2. Example Solution Volume Calculations

Mobile Phase solution		
Operation	*Calculation*	*Volume (ml)*
Prime and purge	20 mL	20
Increasing flow rate	0.75 mL/min * 5 min	4
Initial equilibration	15 CV = 15 * 3 mL	45
Assays	N * flow rate * run time = 4 * 0.75 mL/min * 15 min	45
Syringe cleaning	10 mL	10
Re-equilibration	N * 5 CV = 4 * 3 mL	12
Reserve volume	100 mL	100
Subtotal	Sum of the above	236
Minimum volume	Subtotal * 120%	**284**

Storage solution		
Operation	*Calculation*	*Volume (ml)*
Prime and purge	20 mL	20
Increasing flow rate	0.5 mL/min * 5 min	3
Wash	15 CV = 15 * 3 mL	45
Reserve volume	100 mL	100
Subtotal	Sum of the above	168

Montgomery County Community College

340 DeKalb Pike

Blue Bell, PA

Document Number: QCB 9

Revision Number: 0

Effective Date: 17JUL15

Page 12 of 12

SOP: Buck Scientific BLC-30G HPLC Operation

Minimum volume	Subtotal * 120%	**202**

Equation 1. Example Run Time Calculations

$Run\ volume = 15\ CV$
$Column\ volume\ (CV) = 3\ mL$
$Flow\ rate =\ 0.75\ ml/min$

$$Run\ time = \frac{Run\ volume}{Flow\ rate} = \frac{15\ CV\ *\ 3\ mL/CV}{0.75\ mL/min} = 60\ min.$$

Table 3. Example Flow Rates & Pressure Readings for Ultra C8 5µm 250 X 4.6mm Column (Restek)

Mobile Phase Solution	*Flow Rate (mL/min)*	*Pressure Readings (psi)*
50% MeOH/H_2O	0.5	~1450

11. History

Revision Number	*Effective Date*	*Preparer*	*Description of Change*
0	17JUL15	John Buford, Jason McMillan, Jack O'Neill	Initial release