

HPLC Standard Operating Procedure

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This document is intended to be a guide for operating the Garner Lab's HPLC systems and is only intended to provide instructions for users who are already generally familiar with HPLC operation. If you've never used an HPLC before, then you must be trained by a qualified senior member of the Garner Research Group.

1. System Start-up

- *HPLC*

- 1) Turn on the UV detector for the HPLC you plan to use at least 10 minutes before running samples.

The power switch is located on the front of the detector.

- If the detector is already on, but the UV lamp is off, turn the lamp on by pressing "Shift" + "Lamp" two times.
 - If the "System is not calibrated" message is displayed, then the system must be recalibrated as follows:
 - Ensure that the flow cell contains UV/Vis neutral solvent. If you are unsure about this, consult with a qualified member of the Garner lab before proceeding to the next step.
 - If the flow cell does not contain UV/Vis neutral solvent, flush the flow cell. Consult the 2489 Manual for the proper protocol.
 - Manually recalibrate the system by pressing "Shift" + "3" on the instrument panel. The detector should resume normal operation after this.
 - If the system gives you an error message, power the unit off, unplug it for 5 minutes, then plug it back in and turn it on again. A partially open front cover on the UV detector will cause an error upon startup. Repeat the above instructions. If the detector does not calibrate, contact a Garner group member *immediately*.
- 2) Turn on pump power switch(es) located left side of the pump(s) for the same HPLC.
 - Special Instructions for the Waters 515 Pumps: Each pump needs to be configured to be run remotely by the computer. To do this, cycle through the pumps' main menu with the arrow keys until you find the "Mode Local" setting. Press 'Edit/Enter' to change it to "Mode Rem." Then press the 'Run/Stop' button. Repeat this procedure for both 515 pump units.
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 - 3) Turn on computer and login with given password.
 - 4) Open "Empower 2" (icon on computer desktop) to run the HPLC system from the computer.
 - 5) Login with ID and password.
 - 6) Click "Run samples" icon on a new pop-up window.
 - 7) Select project (Select an existing project or create a new project)
 - 8) Select HPLC system. HPLC_right for the 1525 Binary EF system (analytical or preparative) or HPLC_left for the modular system (analytical only)
 - 9) Click "OK." The system set-up ("Connecting to Empower Node 'Proline.')" will take a few minutes.

** if there is a communication problem between the computer and UV detector, reboot the computer.*

- If not using the Advion Mass Spectrometer, ensure that it is not connected to the HPLC system at this point. If using the mass spce, follow the below instructions:
- *Mass Spectrometer (Optional)*
 - 1) After starting the computer (step 3 for starting the HPLC), open "Advion Mass Express"
 - 2) Click the large red button marked "Operate" in the main menu.

- 3) Connect the PEEK tubing from the mass spec to the outlet “split” from the UV detector on the HPLC

** if there is a communication problem between the computer and mass spec, unplug it's USB cable and plug it back in.*

- 4) In the Advion Mass Express software, go to the Ion Source panel. Load the appropriate ion source file.
- 5) Navigate to the Calibration/Tune and load an appropriate tuning file. See the Advion user's manual if you need to create a custom calibration for your analysis.

2. Solvent Phase Transitioning

- It may be necessary to transition the HPLC system from one solvent system to another. Transitioning may be column specific so consult the column's user manual before attempting any of the following procedures. If you do not correctly transition a solvent system you could destroy thousands of dollars worth of equipment so please make sure you understand what you are doing and that you do it correctly.
- In general, you should transition between normal and reverse phase solvent systems by choosing an intermediate solvent that is miscible with both systems, purge the system, and gradually introduce new eluent(s). Note that you should label and separate solvents that are used for eluent and washing to prevent cross contamination.
- Here is an example protocol for moving between an H₂O/MeCN system to an iPrOH/Hex system:
 - a) Disconnect the initial column and seal each end. Proper column storing involves noting, by way of a label on the column, the solvent composition and date at which the column is being stored (ex. Jan 1, 2013 Stored in 90% H₂O/MeCN)
 - b) Attach both solvent inlets to an MeOH intermediate washing solution and run the system for 10-20 column volumes, ensuring that you also flush the injector loop several times (do this by leaving it in the inject position for several loop volumes of solvent, and/or injecting several loop volumes of the transition solvent)
 - c) Place both solvent inlets and place them in a volume of washing iPrOH and run for 5-10 column volumes, also being sure to flush the injector loop
 - d) Attach solvent line “A” and “B” to your eluent solvents (iPrOH/Hex) and run the system through with 5-10 column volumes with each solvent. Be sure to flush the injector loop again.
 - e) Attach column and equilibrate to the solvent composition at which the column was stored.
- Refer to the column transition chart for further details and specific procedures.

3. Prime the HPLC pumps under the following conditions:

- starting the pumps for the first time after it has been idle for more than 24 hours.
- after changing solvents.

The system must be primed to remove air bubbles and for the pump to function properly.

- 1) Locate the pump draw-off valve (handle located on front of the pump) and priming syringe (6 mL plastic Luer-lock syringe).
- 2) Fully depress the priming syringe plunger to remove all air from the syringe, then insert the syringe into the Luer fitting at the center of the draw-off valve.
- 3) Turn the draw-off valve handle counterclockwise about ½-turn to open the valve.

- 4) Withdraw several milliliters of eluent with the syringe ($3 \times 2\sim 4$ mL). Always close the draw-off valve before breaking the seal with the priming syringe. Ensure that no bubbles remain in the line. Expel the eluent of the syringe into a waste bottle

4. Configure the Empower software to run your method.

- 1) Click “Develop method” icon on the window of Empower2 program.
- 2) Click “Create New” icon.
- 3) Click “*PCM*” *ICON*
- 4) Set up the flow, event, and solvent settings
 - Flow Settings:
 - a. Set pump mode to Isocratic or Gradient
 - b. The table that appears below the isocratic or gradient option box is where you will enter you elution method.

*If using a gradient elution method, it is required that you include a short column washing sequence at the end of your analytical gradient. This will prevent unwanted bands from being retained on the column and getting undesirably eluted in the next run.

- Events settings:
 - a. Check “Enable events“
 - b. Click the first row below Time and then decide events and function as shown below.

	Time	Events	Function	Interval
1	0	Event 1	Pulse	0.02

- Solvents
 - a. add solvent names for A and B that correspond to the solvent system you are using
 - Click “*W2489*” *ICON* or “*W2487*” *ICON*
 - a. For setting the W2489 wavelengths:
 - i. Check “Dual” or “Single”
 - ii. Navigate the Channel tab(s) to set wavelengths.
 - b. For setting the W2479
 - i. Enter a description of each channel.
 - ii. If running a single wavelength, check the “enable” box for channel 1. If running dual wavelengths, check the “enable” box for both channels 1 and 2.
 - iii. Navigate the to the Channel tab(s) to set your wavelength
- 5) Save this *Instrument Method* with a new name.
 - 6) Under “Develop Method,” select “New Method.”
 - Set your desired instrument method.
 - Automated data processing and reporting options can be configured here as well. Consult the Water’s Empower2 Manual if you wish to do this (not recommended for small data sets).
 - Save this *Method Set* with the same name as your *Instrument Method*.
 - 7) In the main Empower window enter the following into the appropriate fields:
 - a. Sample name: put your sample number/ID. It is advised your first injection of the day be a blank sample to obtain a spectrum background.
 - b. Function: Inject samples.
 - c. Method set: select your *method set* which you created following above the procedure

- d. Injection volume: doesn't matter.
- e. Run time: same total time as your *Instrument Method*

5. Configuring the Advion Mass Express software to run in tandem with the HPLC (optional)

- 1) Navigate to the Manual Run tab.
- 2) Load a previously saved method or create your own as follows:
 - a. Check the "Start on input" checkbox. Ensure that the "Low to High" radial button is selected.
 - b. Enter an m/z range for your analysis
 - c. Enter a time interval for acquisitions

*The mass spec has a limit to how many scans over time it can do. If it gives you an error message after the above step, either raise the time interval value or lower the m/z range for your analysis

6. Preparation of a sample for analytical HPLC

- 1) Prepare a ~50 μM solution using HPLC solvent or other appropriate solvent
- 2) If the sample or solvents have not been previously HPLC chromatographed, filter the sample solution through a 0.2 μm filter. You may not inject unfiltered samples.
- 3) Use volatile buffers ONLY. Addition of salt to the instrument will eventually cause precipitation, and additional maintenance. Consult the manuals.

7. Injection

- 1) Click "monitor", and wait for the detector to show a steady, flat base-line and the HPLC pressure reading to become steady. If this does not happen, there is a problem with the system and someone from the Garner lab should be consulted. Double check connections for leaks.
- 2) Click "Abort" (Large Red Button Icon).
- 3) If running the Mass Spec, also do the following:
 - a. Hit the large play button under the Manual Run tab.
 - b. Enter your sample ID into the appropriate field. Make note of both fields in this window – that is where you will find the data the mass spec records.
 - c. Click "OK"
- 4) Click the "Injection" icon button (under the method set drop-down menu). Wait until you get a message "Waiting for injection" at the bottom of the window.
- 5) Take up your sample HPLC syringe. Use either a minimal volume (>25% loop volume) or a volume larger than the sample loop.
- 6) Put the syringe into the injector (slowly until when you feel the end. If you roughly push it, you can scratch inside of the injector, causing damage)
- 7) Gently depress the syringe plunger (don't bend the thin metal!), remove the syringe, then slowly rotate the injector knob from the LOAD position to the INJECT position. The knob rotation must be slow and deliberate otherwise it might not work properly. Make sure that the message is changed from "Waiting for injector" to "Running injection" (takes about 6 seconds).
- 8) After getting the background result, click "monitor" to watch the flat baseline for 2-4 min on the screen.

- 9) Once the baseline is flat, it's time to inject your sample following the same procedure for the background injection. Make sure to enter your sample ID instead of "blank"

8. Washing

- After using HPLC, wash the column and injector systems. This procedure will vary depending on the nature of samples you were running. The procedure below is general for samples were mixtures of normal small molecule organic compounds. If your sample may contain compounds that are unusual or contained metals, a special cleaning procedure may be required.
- If using aqueous buffers, remove all bases, buffer salts, or acids by washing with the washing solution (10% MeOH/H₂O). Clean the injector by filling the sample loop with the washing solution and turning the port from load to inject (this is "flushing the injector"). *If the buffers contained phosphate, leave any phosphate contaminated pump on 0.05 mL/min with pure water overnight. Try to avoid using phosphates at all costs.*
- If using normal phase solvent, flush the injector with iPrOH while washing the column with 100% iPrOH
- If using MeOH or MeCN in water, flush the injector with MeOH while washing the column with 100% of MeOH or MeCN.

9. Shut-down

- HPLC_Left
 - 1) Turn off the modular pumps and UV detector
- HPLC_Right
 - 1) Turn the UV detector lamp off by pressing "Shift" + "Lamp" twice.
 - 2) Optional: Turn the pump and UV detector off. Only do this if asked to.
- Advion Mass Spec
 - 1) Turn off the solvent flow to whichever HPLC it was connected to.
 - 2) Release the reference valve on this HPLC until pressure reads 0. Close the reference valve tightly again.
 - 3) Click the large button labeled "Standby" in the Advion Mass Express software.