CEM Liberty Blue Peptide Synthesizer Standard Operating Procedures

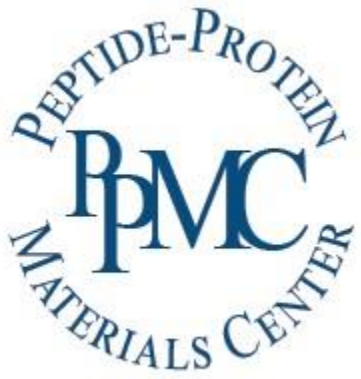


Table of Contents:

About 2
Point of Contact 2
Instrument Location 2
Preparation Checklist 3
A. Preparing Amino Acid and Coupling Reagent Solutions 4
B. Setting Up and Running Syntheses 6
C. Creating a New Liberty Method 8
D. Shutdown 10
E. Troubleshooting 12
F. Generating a Method Report 14
G. Generating a Run History Report 14
H. Recommended Consumables 15
I. Facilities Use Acknowledgements 16

About

The CEM Liberty Blue Peptide Synthesizer features a 4-minute cycle time along with a 90% solvent reduction, based on high efficiency solid phase peptide synthesis. This peptide synthesizer features 27 amino acid positions that can hold up to 120-mL per position. The instrument uses technology for applying microwave energy to both the coupling and deprotection for controlled peptide synthesis at elevated temperatures. This leads to synthesis times under five hours for 30 residue peptides.

Point of Contact

Primary Contact: William Rears

Email: wrears@udel.edu

Instrument Location

Ammon Pinizzotto Biopharmaceutical Innovation Center

590 Avenue 1743, 2nd Floor, Room 255

Newark, DE 19713

Note: Swipe access to the building, floor, and lab are required. Contact Joe Chubbs (jrchubbs@udel.edu) to coordinate swipe access.

Preparation Checklist

Before Your Scheduled Synthesis Time Slot:

1. Use the Usage Calculator to determine the amount of solvent, resin, coupling reagent, and amino acid solutions required for the synthesis.
2. Prepare amino acid and coupling reagent solutions.
3. Weigh out required resin.

Right Before the Synthesis:

1. Fill out the sign-in sheet.
2. Check that the waste container is not full.
3. Ensure there is enough main solvent and deprotection solution for the synthesis.
4. Load amino acids and coupling reagents.
5. Load resin into the reaction vessel.
6. ***Ensure the fiber optic probe is properly inserted into the thermowell.***
7. Begin the synthesis.

When You Are Done:

1. Unload resin from the reaction vessel.
2. Backflush the amino acid and coupling reagent positions.
3. Unload amino acid conical tubes and coupling reagent bottles and dispose of extra reagent. Replace the amino acid conical tubes with the “Empty” amino acid conical tubes.
4. Fill up the Deprotection Solution Bottle.
5. Fill up the Main Solvent Bottle.
6. Check and record the number of temperature errors in the Run History.
7. Logout. If the last user for the day, Shutdown.

A. Preparing Amino Acid and Coupling Reagent Solutions

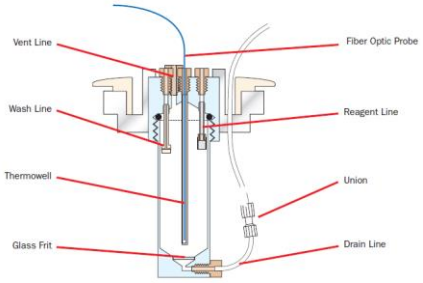
1. Turn on the laptop and open the Liberty Blue software.
2. If the sequence and method already exist for the desired synthesis, go to the **Calculators** menu, and select **Usage Calculator** at the top of the screen.
    a. If the method does not already exist, skip to **section C** to create the method.
3. Under the **Usage Calculator**, select the sequence(s) that will be synthesized from the bottom left **Methods** box.
    a. Sequence(s) can be selected by right clicking the method to be included in the calculation and selecting **Add to Calculation**.
    b. The method will then appear in the table at the top left of the **Usage Calculator**.
4. Write down the required solvent, reagent, amino acids, and resin for the synthesis.
    a. **Solvent** – Each synthesis requires a wash solvent, which is N,N-Dimethylformamide (DMF).
    b. **Reagent** – Both a "base" and an "activator base" are required for the amino acid couplings.
        i. **Base:** 1.0 M Oxyma Pure in DMF
            1. It is recommended that users round up from the calculated required volume of base.
            2. The mass of Oxyma Pure that needs to be weighed out can be calculated by multiplying the total volume required by **0.1421**. That mass will result in a 1 M solution.
                a. ***Oxyma Pure should be weighed in the hood with proper personal protective equipment (safety glasses, gloves, lab coat, pants, closed-toe shoes).***
        ii. **Activator Base:** 1.0 M N,N'-Diisopropylcarbodiimide (DIC) in DMF
            1. It is recommended that users round up from the calculated required volume of activator base.
            2. The volume of DIC that should be measured can be calculated by multiplying the total volume required by **0.1547**. That volume, with DMF added so that the required volume is reached, will result in a 1 M solution.
    c. **Amino Acids** – For syntheses 0.25 mmol or less, amino acids are prepared at a 0.2 M concentration in DMF.
            1. The Usage Calculator calculates the mass of amino acid and volume of DMF required to make a 0.2 M solution.
            2. For non-standard amino acids, such as Fmoc-Lys(Alloc)-OH, this calculation must be done manually. From the required volume in milliliters, this can be converted to liters by dividing by 1000 and then multiplying by 0.2 (concentration required) and the molecular weight of the protected amino acid to yield the mass in grams that must be weighed out.

    d. **Resin** – Based on the loading of the resin, the Usage Calculator determines how much resin is required for a specific synthesis scale.
        i. ***Before running the Usage Calculator, users should ensure that the loading (in mmol/g) of the resin being used matches the loading specified in the synthesis method.***
            1. This will alter the amount of resin that must be added to the reaction vessel.
5. Take the required amino acids out of the freezer to defrost. Wait 15 minutes.
6. Weigh out each amino acid into a labeled CEM conical tube. This can often be done days in advance of booking the Liberty Blue peptide synthesizer. Also weigh out the Oxyma Pure.
7. On the day of synthesis, add the required amount of DMF to the specific amino acid conical tube to dissolve the amino acids. Additionally, add the correct amount of DMF to the Oxyma Pure.
    a. DMF can be measured in a graduated cylinder, and the DMF and Oxyma Pure can be combined and mixed in the reagent container attached to the instrument.
8. Using the same graduated cylinder that was used for measuring DMF, measure out the volume of the DIC. Add DMF to this graduated cylinder to reach the required final solution volume.
9. Weigh out the required amount of resin needed for the synthesis using a weighing boat.
    a. Amino acids, coupling reagents, and resin are now ready for synthesis.
10. Proceed to **Section B** to set up and run the synthesis.

B. Setting Up and Running Syntheses

1. After preparing your amino acids, reagents, and resin, ensure that both the Liberty Blue and Discover Microwave units are powered on via the two power switches on the right-hand side of the machine.
2. Turn on the laptop and open the Liberty Blue software.
3. Check the waste container to the left of the instrument to make sure there is enough room for waste from the synthesis.
4. Ensure that there is enough deprotection solution, which is 20 % piperidine in DMF, and pure solvent, which is DMF, in their respective solvent bottles to the left of the instrument.
    a. If there is not enough deprotection solution or pure solvent, the bottle must first be depressurized:
    b. For changing the deprotection solution:
        i. From the **Options** menu, select **Maintenance**.
        ii. From the **Change Bottle** folder, select the **Change Bottle Deprotection** operation and click **Run Operation**.
        iii. The **Change Bottle** window will appear. Follow the onscreen instructions to depressurize and remove the existing bottle.
        iv. Add the required volume of deprotection solution to the bottle. Ensure the reagent line has a main solvent filter in place, then connect the bottle to the cap.
        v. Click **Next** to continue with the **Change Bottle** procedure. The Liberty Blue will automatically pressurize the bottle. It is recommended that the lines be primed.
    c. For changing the main solvent:
        i. From the **Options** menu, select **Maintenance**.
        ii. From the **Change Bottle** folder, select the **Change Bottle Main Solvent** operation and click **Run Operation**.
        iii. The **Change Bottle** window will appear. Follow the onscreen instructions to depressurize and remove the existing bottle.
        iv. Add the required volume of main solvent to the bottle. Ensure the reagent line has a main solvent filter in place, then connect the bottle to the cap.
        v. Click **Next** to continue with the **Change Bottle** procedure. The Liberty Blue will automatically pressurize the bottle. It is recommended that the lines be primed.
5. Load all the amino acid solutions on to their proper positions on the Liberty Blue. Be sure that empty containers are connected for unused amino acid positions.
6. Load the coupling reagents, DIC and Oxyma, to the Liberty Blue.
    a. It is important that users do not overtighten these bottles. They should be lightly tightened with one hand.

7. To load the resin into the reaction vessel, begin by removing the reaction vessel from the instrument:
    a. To remove the reaction vessel from the microwave cavity, turn the attenuator counterclockwise and lift out the reaction vessel.
    b. Then, disconnect the vessel using the quick disconnect fitting.
    c. Disconnect the vessel body from the attenuator by unscrewing the reaction vessel.
8. Once the RV has been removed from the instrument, the weighed-out resin can be added to the RV. It helps to use DMF to ensure that all the resin is added to the RV.
    a. Rinsing the sides of the RV helps ensure that all the resin is at the bottom of the RV.
9. Verify that the fiber optic probe (blue) is fully inserted into the thermowell.
    a. ***If the probe is not inserted all the way to the bottom of the vessel, the Liberty Blue will not accurately measure the temperature. Overheating of the vessel can occur and will result in poor synthesis quality and/or serious damage to the instrument.***



10. Install the RV back on the instrument by reconnecting the reaction vessel to the attenuator, securing the quick disconnect fitting, and placing the reaction vessel back into the microwave cavity.
        i. ***The quick disconnect only needs to be tightened approximately a quarter turn. Do not overtighten this fitting as this will damage the drain line and cause the reaction vessel to leak.***
11. Under **Maintenance**, find the **Drain** function and drain the reactor. Check that the reactor has been drained when this operation has been completed.
12. From the software main screen, open the appropriate folder in the **Methods** tab to locate the method to be run.
13. Select the desired method by dragging the method to the resin position. Alternatively, users can right-click on the desired method and select **Queue for run**.
    a. If a method has not been defined, see **section C** for instructions on creating a method.
14. The synthesis can now be started. Select the **Start** button to begin the synthesis.
    a. Be sure to check that the pressure has stabilized, and that the synthesis has begun.
    b. When the synthesis has officially begun, an estimated time for the synthesis will be displayed in the lower right-hand corner of the window.

C. Creating a New Liberty Method

1. Begin by opening the appropriate folder or subfolder in the **Methods** box for the new method.
    a. To create a new folder, make sure a Top-Level folder is highlighted, then right click, and select new folder.
2. Click the **+ New Method** button in the lower left corner.
3. The blank method will then appear in the appropriate folder. Enter a name for the method and press Enter.
4. Click into the **Sequence** box. A list of the amino acids in the current bottle setup will drop down. To enter the sequence, either click the appropriate amino acid positions in the drop down or type the one-letter abbreviation for the desired amino acids in order from N-terminus to C-terminus.
    a. If using non-natural amino acid positions, the external number (1-7) corresponding to where the vial is on the Liberty Blue should be used.
    b. To delete an amino acid from the sequence, press the backspace key (to delete the residue before the cursor) or click the delete button on the drop down (to delete the highlighted residue).
    c. To insert an amino acid into the sequence, click an amino acid in the sequence to highlight it. Then either click the desired amino acid in the drop-down or type the one-letter abbreviation for the desired amino acid. The new residue will be inserted before the highlighted amino acid.
5. The following remaining options can then be selected:
    a. Select the scale of the synthesis from the **Synthesis Scale** box drop down menu.
    b. Select which functionality (**acid** or **amide**) will be on the **C-Terminus**.
        i. This is used for calculating the molecular weight of the peptide and not the actual synthesis.
        ii. If using Rink Amide resin, then **amide** should be selected.
        iii. If using Wang resin, then **acid** should be selected.
    c. Select if the **Resin Type** is preloaded or not.
        i. If using Rink Amide resin, it is typically **Not preloaded**.
        ii. If using Wang resin, it is recommended that **Preloaded** resin be used. If preloaded is selected, the program will assume that the amino acid on the C-terminal amino acid is already on resin, and it will begin the synthesis from the second residue.
    d. Select the **Resin Swelling** (**Standard** or **High Swelling**) from the **Resin Swelling** box.
        i. **High Swelling** is commonly used.
    e. **Resin Name** can be entered in the specified box but will not affect the synthesis conditions.
        i. This is an optional parameter.
    f. **Resin Loading** should be entered in the specified box.

        i. Ensure that this value matches the loading on the label of the resin container being used.
        ii. This will not affect the synthesis conditions but will be used in the **Usage Calculator**.
    g. The **Resin Cycle** should adjust based on the scale and resin swelling that are selected.
    h. The **Final Deprotection Cycle** should then be selected. By default, the N-terminal protecting group is removed at the end of the synthesis. Selecting an alternate **Final Deprotection** cycle allows control of this step.
        i. The default is a final deprotection removal of the N-terminal Fmoc.
6. Once the top parameters have been specified, users can assign a cycle to each residue if the default cycle will not be used.
    a. From the **Amino Acid Cycles** table, click the amino acid to highlight it. Then, double click the cycle to open the drop down of amino acid cycles.
    b. To change the cycle for all amino acids to the selected cycle, right-click the cycle and select **Apply this cycle to all**.
    c. **Double Coupling (HS)** is typically employed for most residues.
    d. Users can define new cycles using the **Cycle Editor**. Microwave methods can also be defined using the **Microwave Editor**. ***Users must consult with the individual who oversees the instrument to use these functionalities.***
7. When the method is complete, click **Save** and close the **Method Editor**.

D. Shutdown

This shutdown routine should be performed after every synthesis.

1. Once the synthesis has been completed, begin by removing the reaction vessel from the microwave cavity and removing the resin from the reaction vessel:
    a. To remove the reaction vessel from the microwave cavity, turn the attenuator counterclockwise and lift out the reaction vessel.
    b. Then, disconnect the vessel using the quick disconnect fitting.
    c. Disconnect the vessel body from the attenuator by unscrewing the reaction vessel.
    d. To remove resin from the reaction vessel, use a DMF squirt bottle to suspend the resin and pour it into another container.
    e. Make sure there is no remaining resin on the thermowell or resin disruptor.
2. Once the resin has been removed from the reaction vessel using DMF, rinse the reaction vessel two more times with DMF before reconnecting the reaction vessel to the attenuator, securing the quick disconnect fitting, and placing the reaction vessel back into the microwave cavity.
3. Perform a backflush on all the amino acid, Activator, and Activator Base positions used during the synthesis.
    a. In the **Options** tab, select **Maintenance**.
    b. From the **Cleaning** tab, check the box next to each position to be backflushed.
    c. Click the **Perform Backflush** button. A warning box will pop up. Verify that empty bottles have been connected and tightened for all positions to be backflushed.
4. After the amino acids have been backflushed, unload the amino acid conical tubes and dispose of extra reagent and DMF that has been added to the bottle.
    a. This waste should be disposed of in a properly labeled waste container.
5. Ensure that the Activator and Activator Base bottles have been emptied into an appropriately labeled waste container.
6. After emptying the amino acid conical tubes, replace with the amino acid conical tube that is labeled “Empty”.
7. Fill up the Deprotection Solution Bottle with 20% piperidine in DMF.
    a. This is done by first depressurizing the bottle. Select **Options** > **Maintenance** > **Change Bottle Operations**. Select **Change Bottle Deprotection**.
    b. When the screen displays **Change Bottle Deprotection**, open the bottle, and fill it with the deprotection solution.
    c. After replacing the cap on the deprotection bottle, select **OK**. When prompted, select **Prime** to prime the lines.
8. In a similar fashion, fill up the Main Solvent Bottle with DMF.
    a. This is done by first depressurizing the bottle. Select **Options** > **Maintenance** > **Change Bottle Operations**. Select **Change Bottle Main Solvent**.

    b. When the screen displays **Change Bottle Main Solvent**, open the bottle and fill with DMF.
    c. After replacing the cap on the main solvent bottle, select **OK**. When prompted, select **Prime** to prime the lines.
9. After ensuring the instrument is ready for the next user, it is important to check that there were no significant temperature errors during the synthesis. Go to the **Run History** tab, as done in **section G**, and scroll through the **Run History** and count the number of temperature errors that occurred during the synthesis. Report these on the log sheet.
    a. ***If there is more than one temperature error, notify the individual who oversees the instrument.***
10. Once the whole shutdown checklist has been completed, close the Liberty Blue software.
11. If you are the last user for the day, shutdown the laptop.
12. Power off the Liberty Blue and Discover component via the two power buttons on the right-hand side of the machine.
13. Finally, it is recommended that users perform washes of their resin after the synthesis before completing subsequent modification and/or cleaving peptide from resin. Using a course fritted peptide synthesis reaction vessel, it is recommended that the following washes be performed shaking for one to two minutes in between:
    a. 3 x DCM
    b. 2 x Methanol
    c. 3 x DCM

E. Troubleshooting

The following section provides information on common errors that may occur when using the instrument, as well as ways to rectify these issues.

Symptom/Error	Rectification
Communication Timeout Error	• Close the Liberty Blue software and restart it. • If the error still occurs, exit the Liberty Blue software, shutdown the computer, turn off the instrument, and wait two minutes before turning on the instrument, waiting 1 minute, and turning on the laptop and opening the software.
Missing Amino Acid Bottle Error	• Ensure all amino acid bottles and the activator and activator base bottles are tight. • Ensure the dip tubes for the amino acid, activator, and activator base bottles are tight. • Replace the amino acid dip tube filter. • If the error still occurs, perform a Leak Check Reagent Bottles and a Leak Check Sensor PS3.
Empty Main Solvent or Deprotection Error	• Replace the empty bottle with fresh reagent. • Ensure the bottle is not cracked. • If the bottle is not empty, ensure bottle tubing is securely connected to the manifold and that the cap is tightly closed. • If the bottle is not empty, replace the main solvent filter. • If the error still occurs, perform a sensor calibration of LS1 (for Main Wash) or LS2 (for Deprotection).
Waste Full Error	• Empty the waste container. • If the waste container is not full, ensure the sensor is not engaged (in the up position).
Nitrogen Pressure Errors	• Verify the nitrogen source is functional. • Verify the pressure setting on the Main Pressure Regulator is between 14 and 18 psi. • Perform a Leak Check, then contact CEM Service.

Reaction Vessel Fails Leak Check	• Verify the tubing connections to the manifold on the front of the Liberty Blue are tight and not cross-threaded. • Verify the vessel body is tightly connected to the attenuator. • Verify the PEEK fitting on the drain line is tightly connected to the bottom of the reaction vessel. • Verify the quick disconnect on the drain line is tightly secured.

When an error occurs and the method has been stopped for more than 30 minutes, it is recommended that users reswell the resin before restarting the method. This is how that is done:

1. Click **Stop** to stop the method. Perform any maintenance (replacing filters or bottles) as needed to correct the error.
2. From the **Maintenance** screen, run a **Drain** operation (located under **Miscellaneous** folder) to ensure the reaction vessel is empty.
3. From the **Maintenance** screen, run an **Add Main Solvent (MS2)** operation (located under the **Addition** folder), adding 10 mL of solvent to the reaction vessel.
4. Allow the resin to swell for approximately five minutes, then run a **Drain** operation (located under the **Miscellaneous** folder).
5. Close the **Maintenance Screen**.
6. To restart the method, from the **Current Run** tab, right-click on the appropriate step (the step the method stopped on) and select **Restart Method**.

F. Generating a Method Report

A **Liberty Method Report** records all settings selected in the **Method Editor** when creating the method. **Liberty Method Reports** are created as PDF files which can be saved and printed to allow for easy recording of experimental parameters. To generate a **Liberty Method Report**:

1. Open the **Liberty Method Editor**.
2. Open the appropriate folder or subfolder in the **Methods** box for the new method. Then click on the method to be reported.
3. Click the **View Report** button in the upper right corner of **the Liberty Blue Method Editor** screen to open the **Liberty Method Report Generator**.
4. Select the options to be included in the **Liberty Method Report**.

G. Generating a Run History Report

The **Run History Report** records each command executed by Liberty Blue software during a run with a date/time stamp. In addition, the **Run History Report** records any system errors that occur, allowing for easy diagnosis and troubleshooting of failed syntheses. To create a **Run History Report**, as well as view the **Run History**:

1. From the **Run History** tab, open the folder for the date on which the method was run.
2. Click the desired run. The run history information will be loaded into the table on the right.
    a. If just viewing the **Run History** after a synthesis, this can be scrolled through, and the number of temperature errors can be recorded on the log sheet.
3. Click the **Generate Report** button.
4. The **Report Viewer** window will open, and the method report will be rendered as a PDF.
5. Click **Save** to save the report.

H. Recommended Consumables

The following products are recommended from Fisher scientific. These can be purchased at a discount through UD exchange or through the chemistry store. The Fisher catalog numbers are also provided below.
N,N-Dimethylformamide (DMF): D119-4
Dichloromethane (DCM): D37-4
Methanol: A412-4
Oxyma Pure: 50-187-7780
N,N'-Diisopropylcarbodiimide (DIC): D0254250G

Piperidine must be purchased through Millipore Sigma. The recommended catalog number is 8222990500.

It is recommended that amino acids and resin be purchased through ChemPep Inc. The recommended resins and amino acids, with their associated catalog numbers, are provided below.

Rink Amide Resin: 151801
Fmoc-Ala-OH: 100101
Fmoc-Arg(Pbf)-OH: 100202
Fmoc-Asn(Trt)-OH: 100302
Fmoc-Asp(OtBu)-OH: 100402
Fmoc-Cys(Trt)-OH: 100502
Fmoc-Gln(Trt)-OH: 100602
Fmoc-Glu(OtBu)-OH: 100702
Fmoc-Gly-OH: 100801
Fmoc-His(Trt)-OH: 100902
Fmoc-Ile-OH: 101001
Fmoc-Leu-OH: 101101
Fmoc-Lys(Boc)-OH: 101202
Fmoc-Met-OH: 101301
Fmoc-Phe-OH: 101401
Fmoc-Pro-OH: 101501
Fmoc-Ser(tBu)-OH: 101602
Fmoc-Thr(tBu)-OH: 101702
Fmoc-Trp(Boc)-OH: 101805
Fmoc-Tyr(tBu)-OH: 101902
Fmoc-Val-OH: 102001
Fmoc-Lys(Alloc)-OH: 101244

I. Facilities Use Acknowledgements

The maintenance of this instrument has been supported by the National Science Foundation through the University of Delaware Materials Research Science and Engineering Center (MRSEC). Acknowledging use of this instrument, and its associated MRSEC support, in your publications is very critical to the success and evaluation of the center. Acknowledgements help us demonstrate the Center's value to the University of Delaware research community and contribute tremendously to our efforts to secure funding for new instruments and services so we can continue, as well as add to, our services and capabilities.

If you use this instrument, then the following acknowledgement statement must be included in related publications:

"The authors acknowledge the use of facilities and instrumentation supported by the National Science Foundation through the University of Delaware Materials Research Science and Engineering Center, DMR-2011824."