Standard Operating Procedure

Use of the CEM Liberty Blue microwave assisted solid phase peptide synthesizer

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Reviewer:

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Background

Introduction

Solid Phase Synthesizers are delicate instruments; however a few simple steps can greatly reduce the chances of a break down.

- The system must always be properly shut down when not in use. I.e. all reagents removed and lines flushed. Crystallization and precipitation of reagents inside the instrument can lead to blocked lines and cracked connections which cause gas and liquid leaks and damaged valves.
- When fitting bottles and tubes to the instrument the threads must always be dry and clean, and for resin containing tubes all particulates removed. Damage to the female threads on the instrument leads to gas leaks and the instrument will not run.
- Cross over of solid support material into the liquid side of the instrument. There must always be inline filters attached to the inlets to the liquid side of the instrument.

Reagents

Resins

For standard synthesis pre-loaded copoly (styrene-1 % DVB), 100-200 mesh (75-150 µm) Wang resins are used. See

Appendix A.

Swelling – Resins will swell in the presence of aprotic organic solvents, a swelling time is accounted for in the method.

Swelling factor (mL/g of resin): DMF 4.7 DCM 5.2

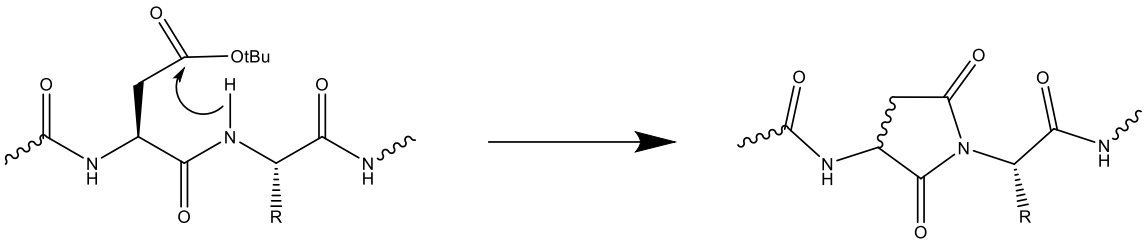
The Reaction Vessel currently installed in the liberty blue is only compatible with 100-200 mesh resins

For more information on resins see: http://www.aapptec.com/resins-solid-phase-peptide-synthesis-core-resins-i-250.html

Sequences which require deviation from this SOP

Aspartimide Formation

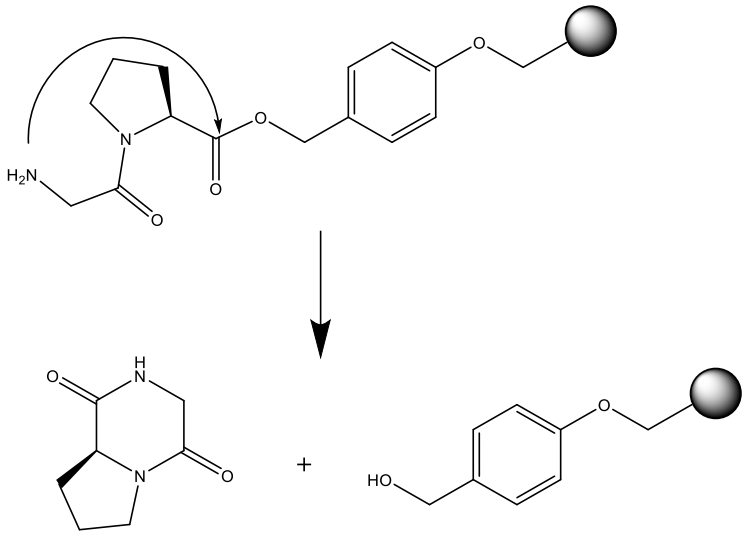
Sequences which contain DN, DD, DG, DT, DS are prone to aspartimide formation.



Aspatrimide can undergo further reactions yielding a mixture of impurities or epimerization of the peptide. Strategies to prevent aspartimide formation are under evaluation and include the use of Fmoc-Asp(OMpe)-OH

Diketopiperazine formation

This reaction can occur during the synthesis of any peptide at the dipeptide stage however sequences containing proline or glycine are particularly susceptible. To this end synthesis of C terminal proline/glycine sequences generally requires the use of sterically hindered solid support.



Tryptophan Alkylation

Wang resins are known to alkylate tryptophan containing sequences – investigation is underway with alternative resins to prevent this.

Alkylation is recognized by a +106 m/z peak.

Standard Operating Procedure

Safety

Please see the separate chemical and instrument risk assessments. These must be read and signed before using the peptide synthesizer.

Read and familiarize yourself with the Safety Data Sheet of all chemicals before use.

Prepare all reagents and solutions in the fume hood, transfer each to the synthesizer with the cap in place.

Calibration

Flow rates and microwave calibration values are determined and set as part of the manufacturer maintenance schedule. Should these need to be checked please refer to the manual.

Equipment set-up

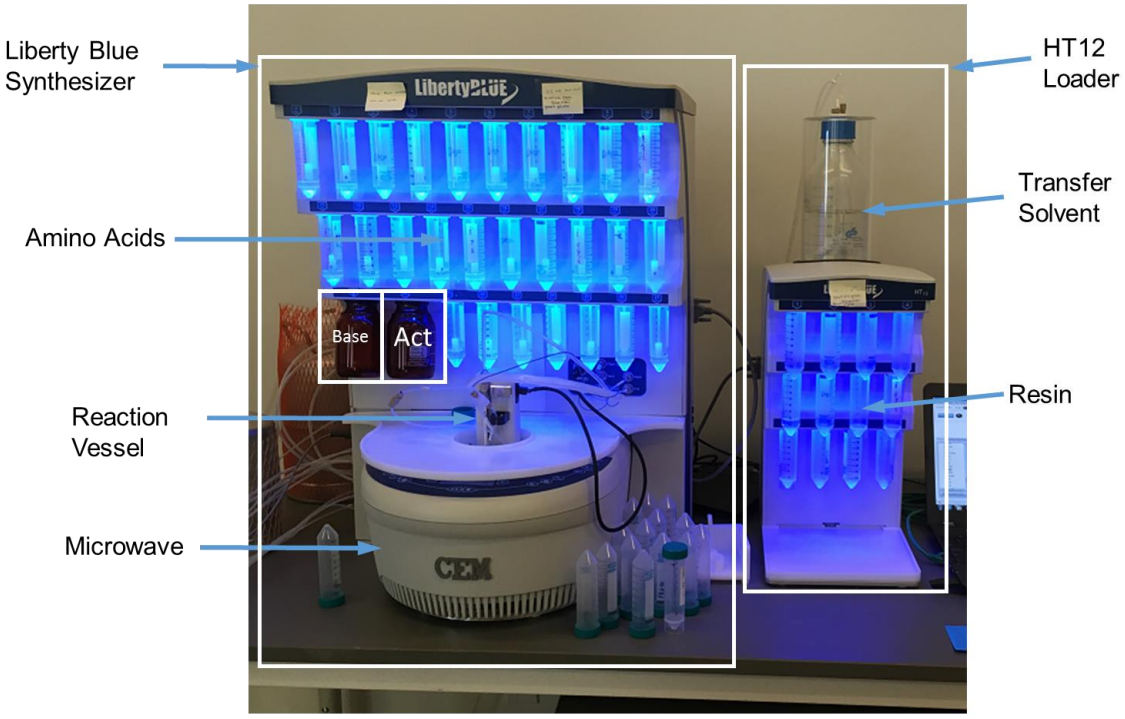


Figure 1: Liberty Blue and HT12 Loader

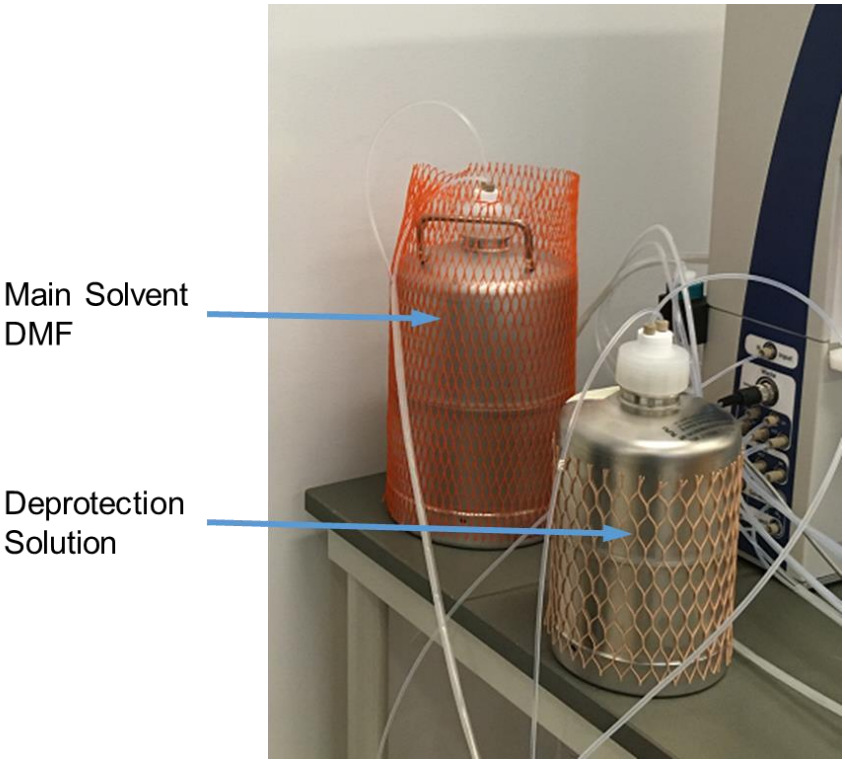


Figure 2: Reservoir Bottles

Powering up the synthesiser

1. Ensure the nitrogen gas supply is on:
    - The wall mounted regulator should read 30 psi (Figure 3)

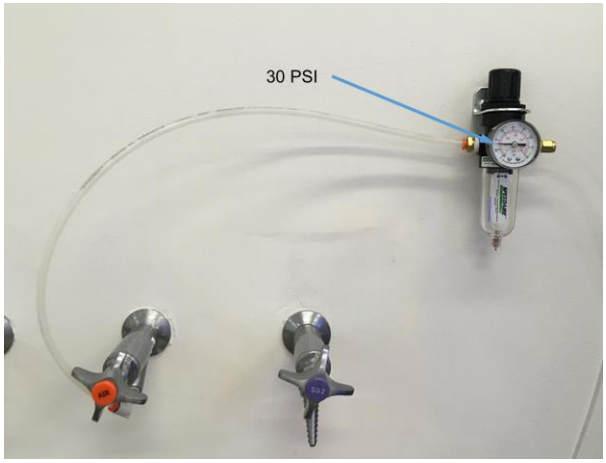


Figure 3: Wall mounted gas regulator. ***Note that this should never normally need adjusted.***

2. Power on the instrument.
3. Turn on the laptop.

Preparing the instrument for synthesis

The first step in preparing for synthesis is to enter your sequences into the software and use the two reagent calculators for preparation amounts.

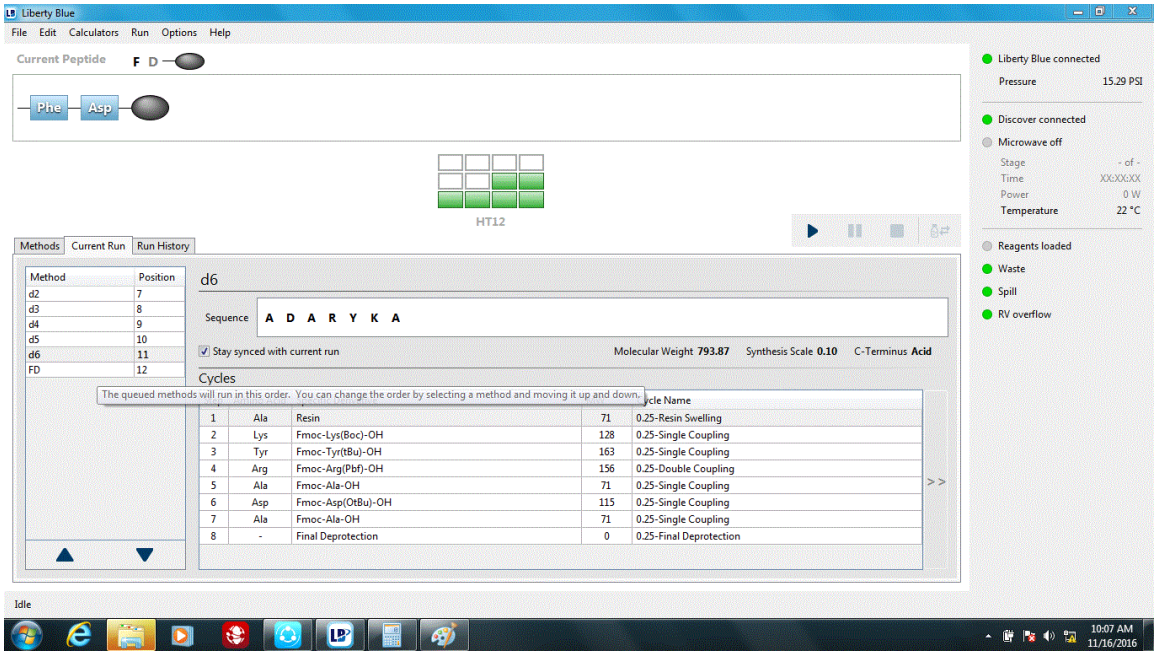


Figure 4. Finished synthesis. Green positions on the HT loader schematic indicate successful synthesis.

4. If someone has recently finished using the synthesizer green positions on the HT12 are completed from the previous completed run. Click on the green box and remove them using the x.
5. Navigate to your folder under the *Methods* tab and open the *Liberty Method Editor…* (Figure 5)

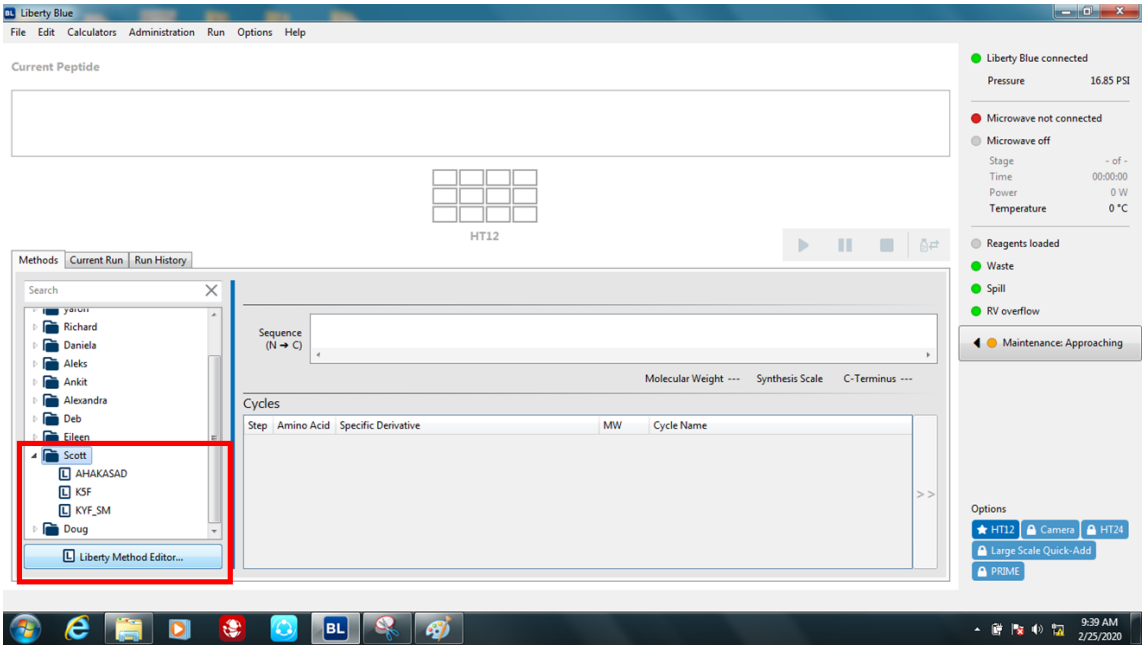


Figure 5. Liberty Method Editor will allow you to add a new peptide sequence.

6. Click the + button to create a new method (i.e. a new peptide sequence) (Figure 6)

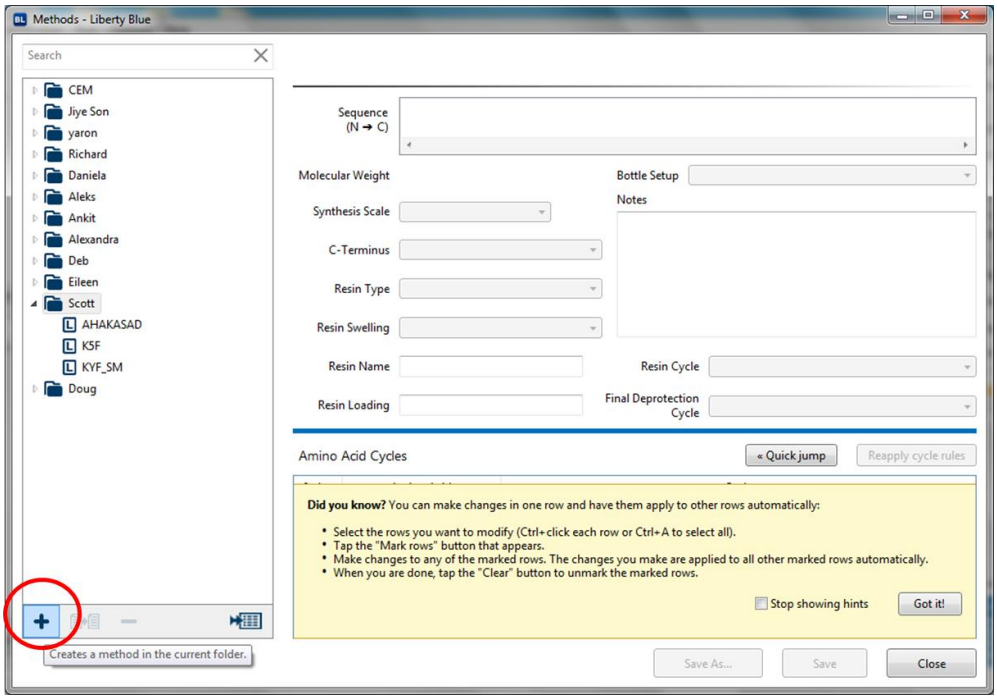


Figure 6. Click the + to create a new peptide sequence

7. Type your peptide sequence into the box and adjust the other synthesis conditions as appropriate. (Figure 7) **Note** that standard conditions are piperidine instead of piperazine as deprotection. The default synthesis parameters currently (Fed 2020) in use within the Ulijn lab are noted in Table 1.

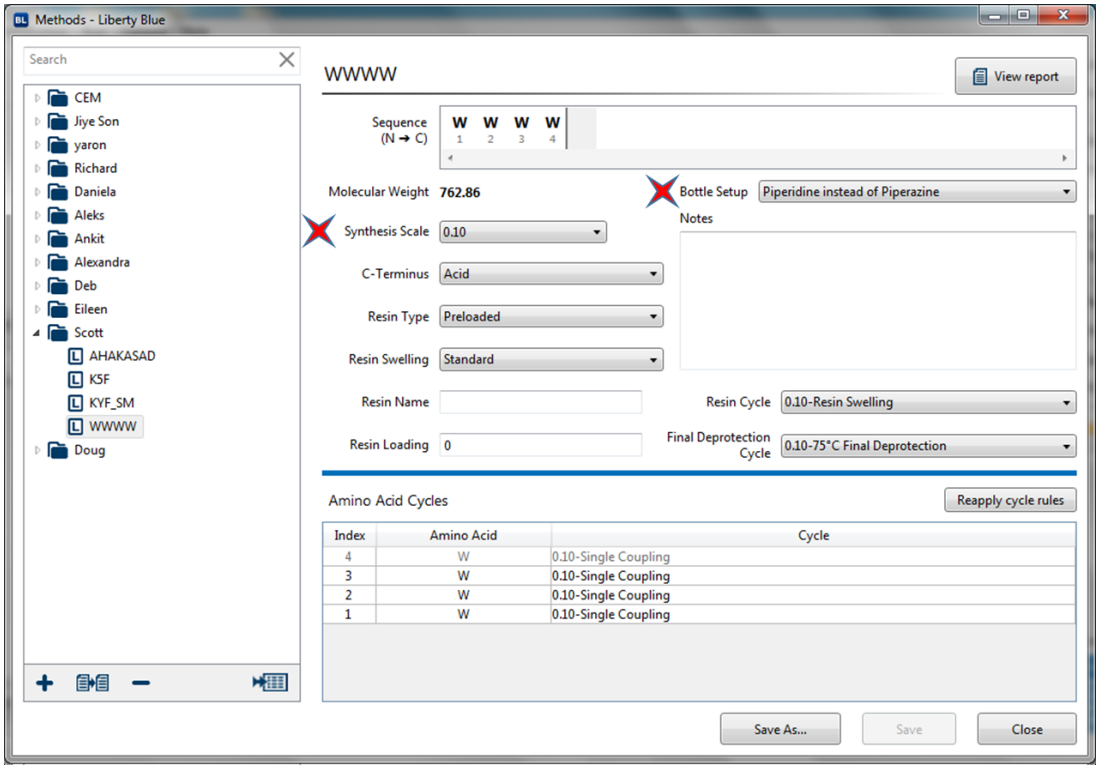


Figure 7. Enter peptide sequence and edit positions starred.

	Reagent
Main Solvent	DMF
Deprotection	10% Piperidine in DMF
Activator	1.0 M DIC in DMF
Base	1.0 M Oxyma in DMF
Fmoc-Amino Acid	0.2 M DMF (OtBu protection strategy)
Resin	Preloaded Wang-type

Table 1: Reagents and Concentrations (See Appendix A for further details)

8. Save your method and repeat for each peptide sequence.
9. Once all sequences are entered go back to the main screen.
10. Drag each peptide sequence to be synthesized during the batch from the method menu into a free position on the loader. (Figure 8). They will turn blue and pulse.

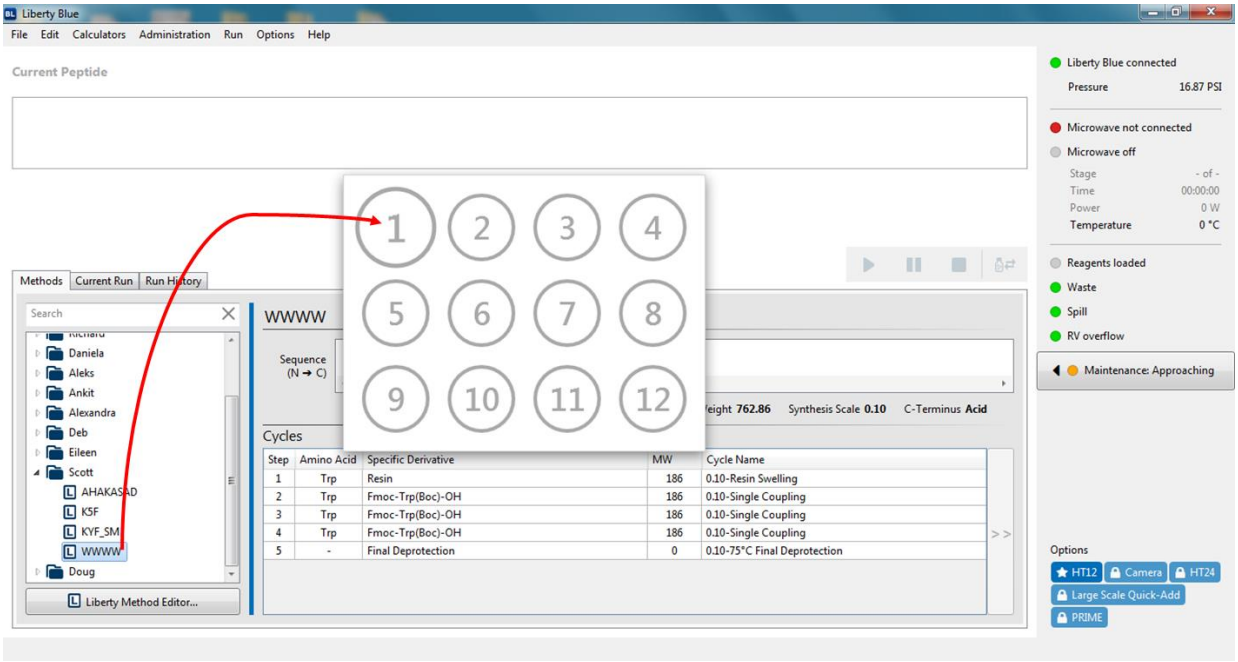


Figure 8. Drag peptide sequence to a position on the HT12.

Reagent Calculators

1. Open the *Calculators* ⇨ *Usage Calculator* and create a report by clicking *View Report*. Each of the peptides to be synthesized in the planned batch should be checked on the list. Be patient, it may take several minutes for this window to respond. It may be convenient to save the report to a USB stick and print it. (Figure 9)

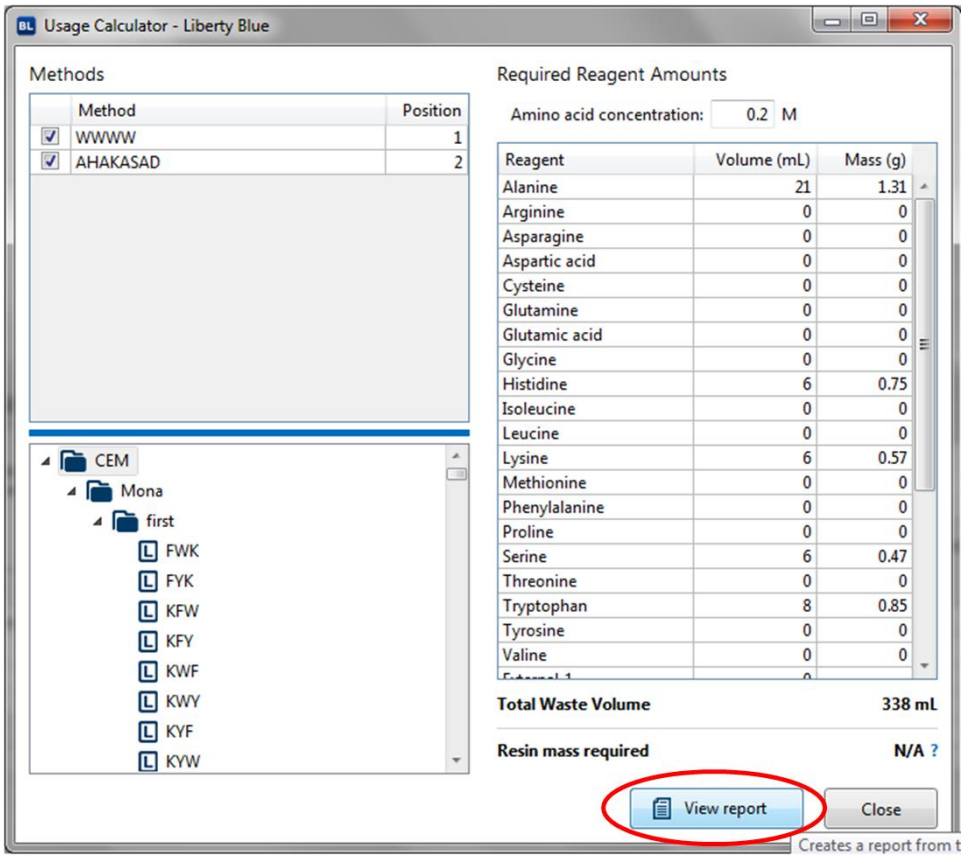


Figure 9. Click View Report *to generate a usage report for this batch of peptides.*

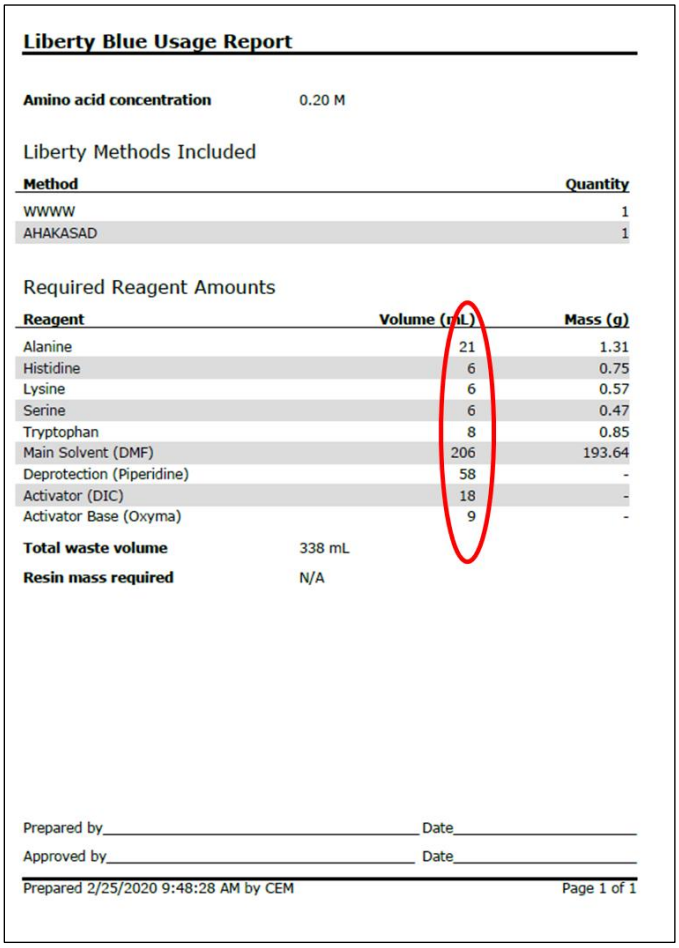
Chemical synthesis will always be most efficient with freshly reagents. Factors such as: exposure to UV light, exposure to atmospheric oxygen and reaction with trace contamination during preparation will all effect the stability of the reagent solutions.

Always write the "Prepared on DATE" and initial when adding a new solution bottle. When topping up a solution bottle do not amend the prepared on date.

Consult the table below for recommended lifetime of reagents

Table 2. Reagent stability under inert atmosphere

Reagent	Stability on the synthesizer (under N_2)
Fmoc- Amino Acid	One week
Activator (DIC)	Two weeks
Base (Oxyma)	One week
Deprotection	One Month



Liberty Blue Usage Report

Amino acid concentration 0.20 M

Liberty Methods Included

Method	Quantity
WWWW	1
AHAKASAD	1

Required Reagent Amounts

Reagent	Volume (mL)	Mass (g)
Alanine	21	1.31
Histidine	6	0.75
Lysine	6	0.57
Serine	6	0.47
Tryptophan	8	0.85
Main Solvent (DMF)	206	193.64
Deprotection (Piperidine)	58	-
Activator (DIC)	18	-
Activator Base (Oxyma)	9	-

Total waste volume 338 mL

Resin mass required N/A

Prepared by_____ Date_____

Approved by_____ Date_____

Prepared 2/25/2020 9:48:28 AM by CEM Page 1 of 1

Figure 10. Usage report for batch of peptides to be synthesized.

The instrument will slightly over use reagents, to compensate and make set up more convenient follow the steps below.

1. Open *Calculators* ⇨ *Reagent Calculator* and navigate the tabs and follow the instructions below for each reagent.

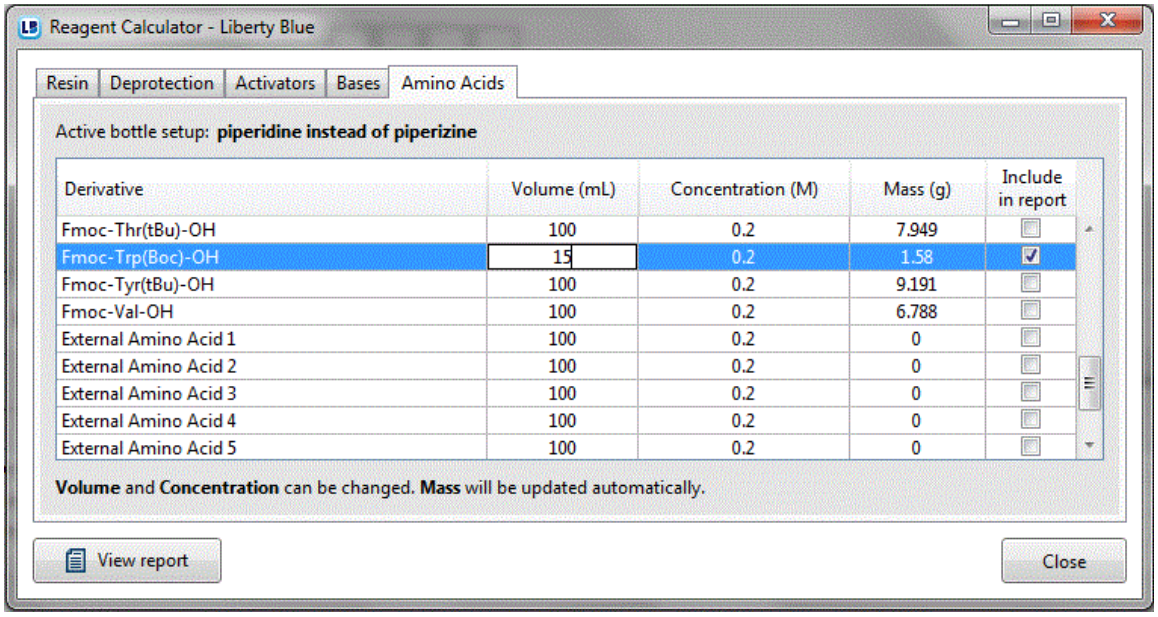


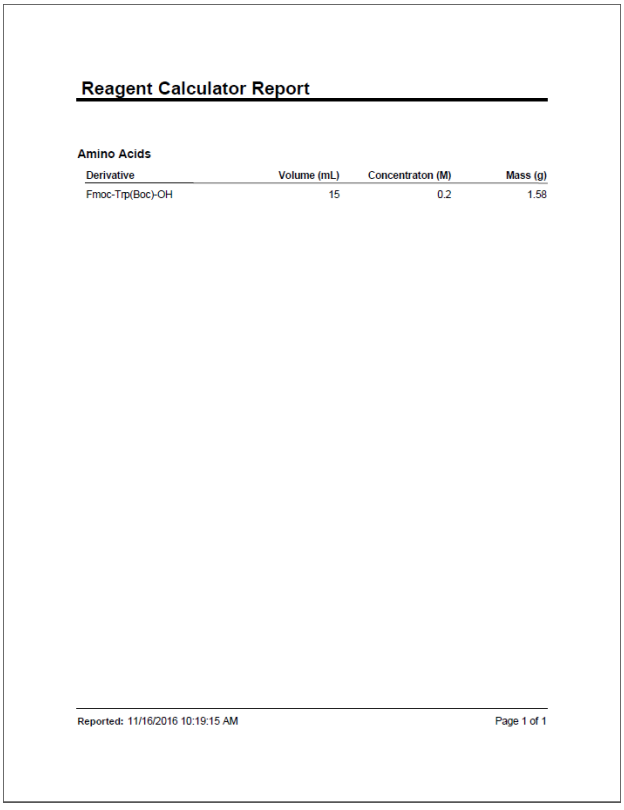
Figure 11. Enter the new value of volume to be prepared.

2. For Fmoc-AAs increase the volume from the **Usage Report** (Figure 10) to be prepared by rounding up to the nearest 2.5 mL. This will allow you to conveniently dispense solvents on the graduations of the 50 mL tube.
3. Ensure there is a minimum of 1 L of DMF in the main solvent bottle.
4. Increase deprotection solution to prepare a minimum of 500 mL. Consult Table 3 below to determine the contents of aluminium bottles. Remember to take the lines out.

	Tare of aluminium bottle	Density of solution
Main Solvent (DMF)		
Deprotection (20% v/v Piperidine/DMF)	1119g	

Table 3: Bulk reagent bottle measurements

5. Prepare a minimum of 25 mL of Activator A
6. Prepare a minimum of 25 mL of Activator A
7. Ensure each reagent required in the Usage Report has the checkbox for "*add to report*" checked.
8. Generate report using *View Report* (Figure 12). It may convenient to save this report to a USB stick and print it.



Reagent Calculator Report

Amino Acids

Derivative	Volume (mL)	Concentraton (M)	Mass (g)
Fmoc-Trp(Boc)-OH	15	0.2	1.58

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Figure 12. Reagent calculator report. Note that this is example only have one value for an adjusted amino acid.

Resin Calculation and Preparation

Figure 13: Pre-loaded resin with substitution stoichiometry indicated

The solid support resin is difficult to weigh out due to the effect of static electricity.

1. Remove the desired resin(s) from the fridge.
2. Open *Calculators* ⇨ *Reagent Calculator* ⇨ *Resin*

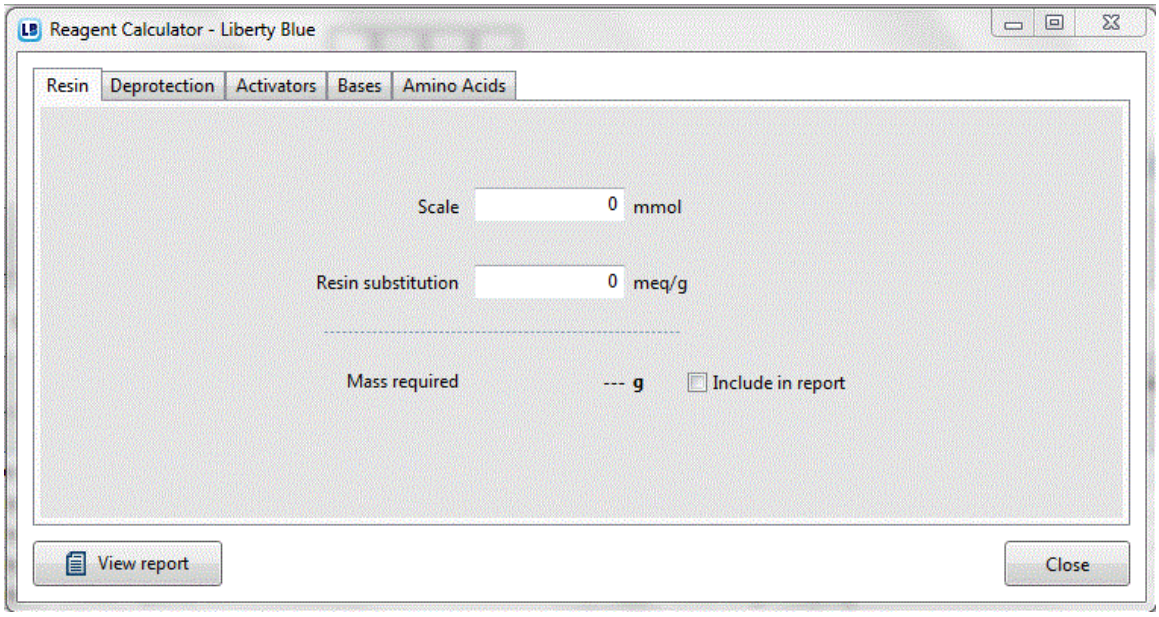

Figure 14. Calculator the mass of resin to be weighted out

3. Enter the synthesis scale chosen in your method and the loading stoichiometry (mmole/g) from the resin bottle (Figure 13), note the mass of resin required. Repeat for each sequence to be synthesized.
4. Weigh the resin(s) into a new, clean 50 mL tube. Do not to weigh out on the balance plate but on the bench.
5. Add 10 mL DMF to the tube.
6. Attach the tube to the HT12 loader. Be sure to carefully clean away any resin which has accumulated on the thread or lip of tube, it will cause it not to seal properly and fail a gas pressure test.

Fmoc-AA and Reagent Preparation

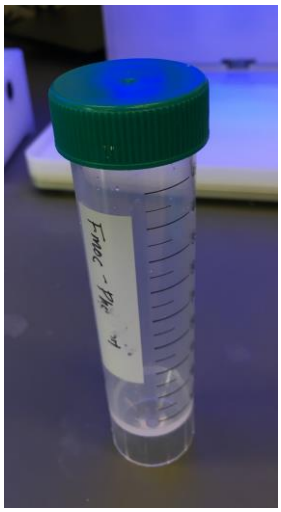

Figure 15: Labcon branded 50 mL tube. Several types are available within the lab: skirted with standard caps, and unskirted with gasket caps or standard caps.

The synthesiser will only accept Labcon branded 50 mL tubes (Figure 15**), others do not seal and cause will cause the instrument to fail pressure tests.**

1. Prepare Fmoc-AA solutions in new 50 mL tubes. If your batch of peptides requires more that 40 mL volume use a large format AA bottle found in cabinet under synthesizer. (Figure 16)

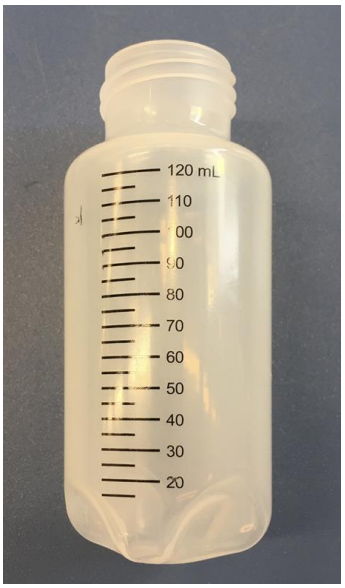

Figure 16. Large format Fmoc-amino acid bottle

2. Prepare activator solutions in new 50 mL tubes or clean amber 250 mL Duran bottles depending on the volume required.
3. Prepare deprotection solution in a measuring cylinder which has been triple rinsed with DMF.

The Main Solvent and Deprotection Solution bottles are under pressure and must be depressurized in the software before removing the cap.

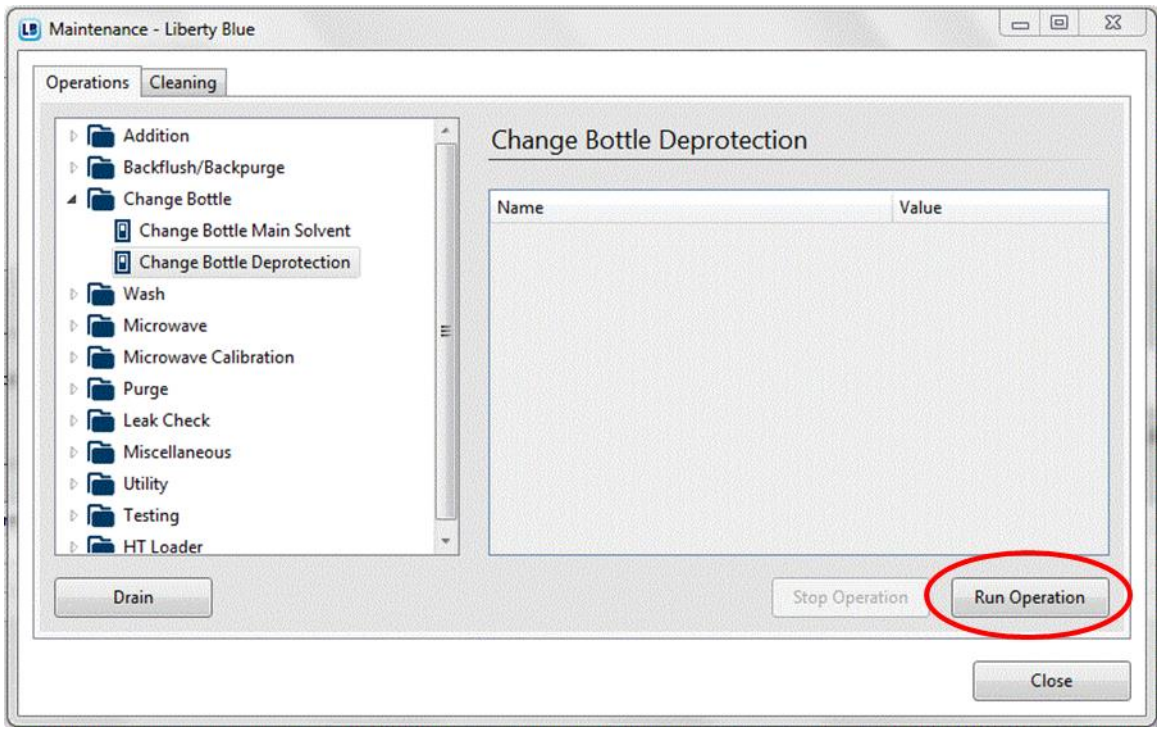

Figure 17. Changing bottle operation

4. First add Main Solvent DMF to the synthesiser: *Maintenance* ⇨ *Operations* ⇨ *Change Bottle Main Solvent* ⇨ *Run Operation*
    a. When prompted by the software remove the cap and lay the line on a clean lint free paper tissue.
    b. Inspect the filter for discoloration and replace if necessary.
    c. Add fresh DMF to the bottle in the fume hood. Wipe the thread and rim of the bottle with a lint free Kimwipe to ensure a good seal.
5. To add deprotection solution follow steps a – c above with the *Change Deprotection Bottle* option.

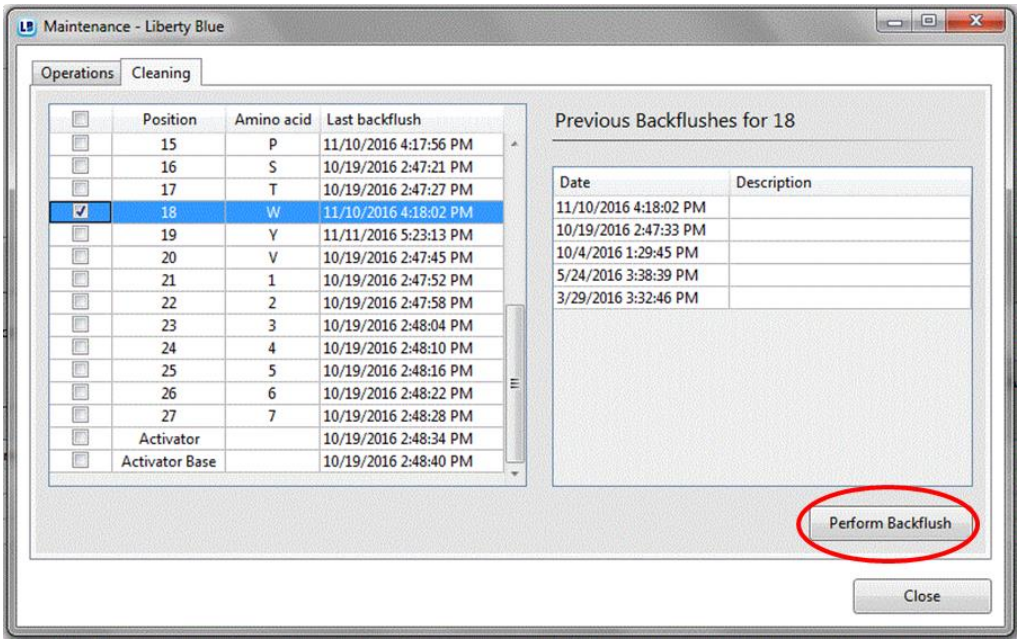

Figure 18. Backflushing cleans the lines with DMF and nitrogen before adding fresh solutions.

6. To add Fmoc-AA's to the synthesiser
    a. Open the *Options* ⇨ *Maintenance* ⇨ *Cleaning* and check the positions of AA's to be added. Click *Perform Backflush*. This will clean the line with a little DMF and flush the line with nitrogen. (Figure 18)

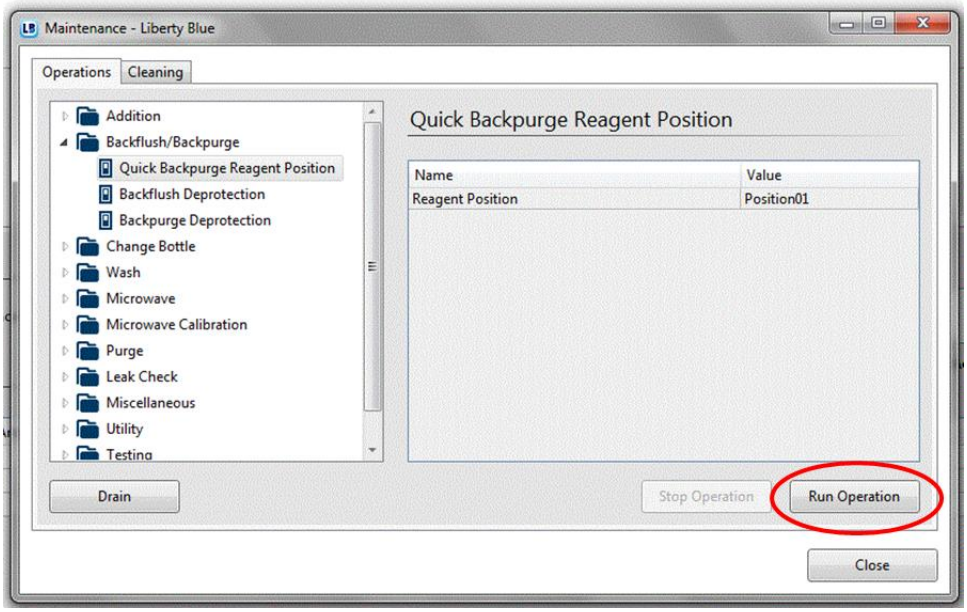

Figure 19. Quick backpurge will blow nitrogen gas through the line to clear it of liquid.

   b. Open *Operations* ⇨ *Quick Backpurge* back purge each position and add the AA tube in turn. (Figure 19)
7. To add Activator solutions, repeat steps above with the appropriate boxes checked.
8. Top up the Transfer Solvent bottle with 50% v/v DCM/DMF, swirl bottle until thoroughly mixed. **Use a measuring cylinder which has been triple rinsed with DCM to prepare this solution.**

Starting the Synthesizer

1. All reagents and resins should now be in place
2. Check that there is sufficient space in the waste bottle for the volume indicated in the usage report.
3. Press the play button in the software.

Setting the Synthesizer to Standby

It is vital to the maintenance of the synthesizer that it is set to standby when you have finished using it. Usage will be monitored and the instrument checked after use.

1. Remove Fmoc-AA tubes:
   a. Open *Options* ⇨ *Maintenance* ⇨ *Cleaning* and check the positions of AAs to be removed. Click *Perform Backflush*. This will clean the line with a little DMF and flush the line with nitrogen.
   b. Replace the backflushed tubes with a clean tube and repeat step a. Label the new tube as “DMF”
2. Remove Activators as above.
3. Leave Main Solvent DMF and Deprotection Solution in place.
4. Turn off the power to the microwave unit and HT12. Leave the power on to the Liberty Blue.

The supply of nitrogen does not need to be turned off. If the power is on to the Liberty Blue then all the reagents and bottles will be under inert atmosphere.

Removing Finished Syntheses from the HT Loader

Due to pressure differentials when the instrument is operating solid support will often creep back up the tube. To clear the resin back into the tube:

1. Open *Options* ⇨ *Maintenance* ⇨ *HT Loader* ⇨ *Transfer Solution to HT Position*
2. Change the Volume from 10 to 1
3. Run operation.
4. **Remove the tube containing the resin whilst nitrogen is bubbling.**
5. Replace with a clean empty tube.

Cleavage and Acidic Deprotection

This section of the SOP assumes standard synthesis conditions and use of the vacuum manifold. Suggested volumes are for short peptides and 0.1 mmol synthesis scale, scale up as appropriate.

Table 4: Deprotection Cocktail

	Volume %
Trifluoroacetic acid	92.5
Triisoproylsilane (TIS)	2.5
Water	2.5
2,2′ -(Ethylenedioxy)diethanethiol (DODT)	2.5

1. Ensure there is 10 mL of diethyl ether in the freezer for each peptide to be deprotected.
2. Place a new supelco tip liner in each manifold position to be used.
3. Put a clean tube in the manifold chamber under the positions to be used.
4. Attach a new labelled filter to the manifold and turn on the vacuum.
5. Pour the solution with the suspended resin into the filter. Wash out any further resin with DCM.
6. Wash the resin with 3x 10 mL of DCM. Turning off the vacuum to maximize contact time with the resin.
7. Allow the resin to dry under vacuum for 10 minutes.

8. Make up fresh deprotection cocktail, chill the TFA on ice before adding other constituents.
9. Add the resin to a clean 50 mL tube and use approximately 1 mL of deprotection cocktail per 0.1g of resin.
10. Put the tube on a shaker/rocker, observe that the resin is well suspended in the deprotection cocktail and allow to react for a minimum of 2 hrs room temperature.
11. Filter the resin from the deprotection cocktail using the filter from step 7 into a clean 50 mL tube and wash the resin with 2 mL of leftover cocktail or neet TFA. Retain the resin until you have HPLC confirmation of your peptide. You may aliquot the deprotection cocktail into small or different tubes as per the available equipment.
12. Evaporate the deprotection cocktail until there is approximately 1 mL of orange-ish, viscous solution.
13. Cool the crude solution on ice and add 2 mL of ice-cold diethyl ether. There should be an immediate precipitation.
14. Vortex the solution and cool on ice for 10 minutes.
15. Centrifuge lightly, decant and retain supernatant.
16. Wash pellet 2x with ice-cold diethyl ether, resuspending the pellet and re-centrifuging each time.
17. Dry the pellet in air or under vacuum until it no longer smells of ether.
18. Resuspend the pellet in an appropriate volume of water. If dissolution proves difficult add increasing percentages of acetonitrile or adjust the pH. Peptide is now ready for HPLC analysis.

Principal Investigator or Lab Supervisor SOP Approval

Print Name: Signature: Date:

Appendix A

List of synthesis materials

Table 5: Novabiochem supplied resins and amino acids

AA	Side chain Protecting Group	Resin Product code	Fmoc-AA
Ala	-	856001	852003
Arg	Pbf	856002	852067
Asn	Trt	856004	852044
Asp	OtBu	856005	852005
Cys	Trt	856006	852008
Gln	Trt	856007	852045
Glu	OtBu	856008	852009
Gly	-	856009	852001
His	Trt	856010	852032
Ile	-	856011	852010
Leu	-	856012	852011
Lys	Boc	856013	852012
Met	-	856014	852002
Phe	-	856015	852016
Pro			852017
Ser	tBu	856016	852019
Thr	tBu	856017	852000
Trp	Boc	856018	852050
Tyr	tBu	856019	852020
Val	-	856020	852021

Table 6: Other Synthesis Reagents

	Supplier	Product Code	Note
DMF	Fisher	BP1160-4	Sequencing grade or better required
1-methyl-2-pyrrolidinone	Fisher	AC354900025	Alternative to DMF for longer peptides
Piperidine	Sigma-Aldrich	411027	
DIC	Sigma-Aldrich	D125407	
Ethyl (hydroxyimino)cyanoacetate (Oxyma)	Sigma-Aldrich	8510860100	

Table 7: Cleavage and Acid Deprotection Reagents

	Supplier	Product Code
Trifluoroacetic acid	Sigma-Aldrich	T6508
Triisoproylsilane (TIS)	Sigma-Aldrich	233781
2,2′ -(Ethylenedioxy)diethanethiol (DODT)	Sigma-Aldrich	465178
Water (HPLC Grade)		
Diethyl Ether		

Table 8. Miscellanous materials

	Supplier	Product Code
Labcon 50 mL tubes (Skirted)	Labcon	3095-345