Manual Solid Phase Peptide Synthesis Protocol

(modified 12/14/16)

With FMOC amino acids
Excellent video: https://www.youtube.com/watch?v=jVXUfC2pLh4
https://www.youtube.com/watch?v=9zSrOnMi53M

Homemade synthesizer apparatus: https://www.youtube.com/watch?v=tN12vHxHqEg

Chemistry of FMOC solid phase synthesis: https://www.youtube.com/watch?v=THBpOHZzAR8

For FMOC synthesis make sure that any side chains are protected with groups that are NOT sensitive to base, but rather can be deprotected in the final TFA step.
Ex. Boc protected Ser/Thr
Trityl protected Histidine

Needed:

Poly prep Biorad column
Rink Amide Resin (uses same deprotection step as FMOC deprotection)- starts with an FMOC attached)
1-methyl-2-pyrrolidinone (N-methylpyrolidinone) (NMP) used to swell beads
(Note: DMF is commonly used in place of N-methylpyrolidinone)
Glass vials to mix
Glass test tubes
10 mL Plastic Syringe
Rubber septa (hole drilled) to force solvent out
% 4-methylpiperidine
FMOC protected amino acids (sidechains w/ acid labile protecting groups)
HATU (or HBTU) – for aa activation
DIEA (diisopropylethyl amine)
Kaiser Test Reagents or Anaspec Ninhydrin Test Kit (#AS-25241) 510-791-9560
Metal spatula
Microcentrifuge tube
Heating Block
Vacuum Desiccator
TFA (Trifluoroacetic acid)

****Each Peptide Synthesis will begin by filling out a "Peptide Synthesis SHEET" in <u>BOTH</u> electronic and manual forms.**

The following Peptide synthesis is on a <u>100 mg scale of Rink Amide Resin</u>
(Can be scaled up)

<u>Steps for Manual Solid Phase Peptide Synthesis</u>

1. **<u>Pre-swell Resin (Use Glass 1dram vial)</u>**
    a. Weigh 100 mg Rink amide resin into a glass vial (1 dram vial with sealable cap)
    b. **<u>RECORD</u>** the <u>actual weight of the resin</u> that was used on the Peptide Synthesis sheet (**VERY IMPORTANT STEP FOR FUTURE REFERENCE**).
    c. Add 1 mL of N-methyl pyrrolidone (NMP) (can also use dimethylformamide DMF). (If larger scale, use 1mL for each 100mg of resin)
    d. Let sit overnight in the refrigerator (or at least 8 hours)

2. **<u>Preparation of Column</u>**
    a. Remove the plastic tab on end of BioRad polyprep column
    b. Put on small yellow plastic cap

3. **<u>Load column with Resin Beads</u>**
    a. Clamp the polyprep column using a ring stand and place a small beaker under the outlet (remove yellow cap)
    b. Pour the slurry of pre-swollen beads into the polyprep column
    c. Rinse glass vial with 1 mL N-methyl pyrrolidone (NMP), pour onto column
    d. Apply pressure with Rubber septa and 10 mL plastic syringe to the top of the column to force liquid to flow through.
    e. Repeat steps c and d 1 more time.
    f. (**IMPORTANT**) Place yellow cap back on column.

4. **<u>Deprotection of FMOC groups on Rink Amide Resin</u>**
    a. Make a solution of 20% 4-methylpiperidine (base) in 80% N-methyl pyrrolidone (NMP) in a 15 mL vial. (Add 2 mL 4-methylpiperidine to 8 mL N-methyl pyrrolidone (NMP)) (Can scale up if desired – **KEEP FOR FUTURE REACTIONS**)
    b. Add 2 mL of the above 20% piperidine solution to the beads in the column.
    c. Place larger clear plastic cap on top of column, snap closed.
    d. Agitate by hand then place on vortexer with clamps at medium speed for 15 min.
    e. Take off top column cap 1st, then take off bottom yellow cap.
    f. (**IMPORTANT**) **<u>Collect and save</u>** the deprotection solution in a small test tube (DO NOT USE PRESSURE- allow to drain by gravity until all the solution has drained.)

5. **UV spectral analysis FMOC deprotection (used to quantitate success of FMOC deprotection)**

<u>Note</u>: only do this step for first AA coupling to establish scale of reaction

    a. Place 3 mL (200 proof) ethanol in a 3 mL quartz cuvette as blank.
    b. After blanking the instrument, using a manual pipettor, remove 10 µL of ethanol from the blank cuvette and place 10 µL of the eluate from test tube (step 4f) and

place in remaining 2990 µL of (200 proof) ethanol (**300X Dilution**) in the 3 mL cuvette.

c. Mix with parafilm. Remove parafilm.
d. Place in spectrophotometer and record a spectra between 220 nm and 450 nm. (Alternatively, take a single point read at 300 nm)
e. Record the Absorbance at 300 nm (λ_{max} of FMOC group)
f. Using an extinction coefficient for FMOC of ε=7800 $M^{-1}cm^{-1}$, input ABS_{300} value into Beer's law to calculate concentration of deprotected FMOC.
g. Example calculation: ABS_{300} = 0.495 = conc. x 1 cm x 7800 $M^{-1}cm^{-1}$ = 0.0001905 M. Now multiply by dilution factor x 300 = .01905 M or 19.05 mM. Therefore, my original 1 mL of elution actually has <u>19.05 µmol of eluted FMOC</u>.
h. **<u>RECORD</u>** the result of these calculations on the Peptide synthesis sheet. (**VERY IMPORTANT STEP FOR FUTURE REFERENCE**)

6. Washing the Column Beads (to remove all traces of piperidine)

a. Add **1 mL NMP** with manual pipetor to the column while rinsing down the sides of the column. Collect in small beaker – ultimately can be discarded.
b. Apply pressure with syringe/septum to drain NMP.
c. Repeat steps a-b 2X more. (**Total of 3 washes**)
d. Add **1 mL dichloromethane (DCM)** (CH_2Cl_2) with manual pipetor to the column while rinsing down the sides of the column. Collect in small beaker – ultimately can be discarded.
e. Apply pressure with syringe/septum to drain dichloromethane.
f. Repeat steps d-e 3X more. (**Total of 4 washes**)
g. Discard combined washes in waste.
h. Rinse off yellow cap and larger top snap cap with Acetone (reduces contamination).
i. Dry yellow cap and top snap cap with paper towel.

7. <u>Activate amino acids for attachment to the resin</u>

a. First, make a stock solution of DIEA in NMP (2.0M), (Note: you will add a certain amount of this stock to your activation reaction.)

   <u>To make:</u> To a 15 mL scintillation vial, add 3.484 mL of DIEA to 6.516 mL NMP. (<u>Label and save this vial for other reactions</u>)

b. You must **determine the scale of the reaction** by using the values calculated for the released FMOC group from step 5g above. Example calculation: If your FMOC moles is 19.05 µmol, this would also mean you have the same number of moles of "free amine" on the resin.

Weigh out all the following (except resin) in labeled microcentrifuge tubes.
All of the following solutions will be made with NMP.

*Weigh the AA in 15 mL scintillation vial, add NMP, add flea stir bar.
*Weigh HATU in 1 dram vial, add NMP, mix, transfer to 15 mL scintillation vial that contains the AA.
*Add DIEA, let react 5 min (reaction will only turn pale yellow if you add DIEA)

Reagent	**Mol. Wt. (g/mol)**	**# mol. Equiv.**	**Mol. Req.**	**Mass To weight out**	**Desired Conc. in NMP**	**Volume of NMP to add**
Amines on Resin	na	1	19.05 μmol	na	na	na
HATU	380.2	2.85	19.05 x 2.85= 54.3 μmol	380.2 g/mol x .0000543 mol = 0.0206 g (20.6 mg)	0.2M	0.2715 mL (271.5 μL)
FMOC prot. AA Ex. FMOC-Leu	**Look up** Ex. 353.4 g/mol	3.0	19.05 x 3.0= 57.2 μmol	353.4 g/mol x .0000572 mol = 0.0202 g (20.2 mg)	0.2M	0.286 mL (286 μL)
DIEA (diisopropyl ethylamine)	129.25 d=0.742 g/mL	5.7	19.05 x 5.7= 108.6 μmol	na	2.0M stock solution from above	0.0543 mL (54.3 μL)

c. **RECORD** these calculations on the Peptide synthesis sheet for each amino acid added. (**VERY IMPORTANT FOR FUTURE REFERENCE**)
d. Combine the liquid HATU and FMOC AA solutions in a 15 mL scintillation vial, add the calculated volume of the 2.0 M DIEA stock to this mixture.
e. Place a small flea stirbar in the scintillation vial, place on a magnetic stir plate with gentle stirring, and allow to react 5 min (use timer).
f. Note: this reaction should develop a slight yellow color change.

8. Add activated amino acid to the deprotected beads of the column

a. **Using a glass pipette**, add the activation reaction mixture (from step 7e) to the deprotected beads on the column. (Make sure column still has yellow small cap on.)
b. **IMPORTANT**: Place the top cap back on the column.
c. Re-suspend the beads manually with gentle shaking, place on vortexer with clamps at medium speed for at least 20 min. (Every five minutes, re-suspend beads manually and place back on vortexer). Can be done longer if needed up to 30-60 min more.

9. Wash column to remove unreacted AA, HATU, and DIEA

a. Add **1 mL NMP** with manual pipetor to the column while rinsing down the sides of the column. Collect in small beaker – ultimately can be discarded.

b. Apply pressure with syringe/septum to drain NMP.
c. Repeat steps a-b 2X more. (**Total of 3 washes**)
d. Note: The first eluates should have a more intense yellow color due to the release of hydroxyl azabenzotriazole (HOAt). This color will become less intense with each wash.
e. Add **1 mL dichloromethane** (CH_2Cl_2) with manual pipetor to the column while rinsing down the sides of the column. Collect in small beaker – ultimately can be discarded.
f. Apply pressure with syringe/septum to drain dichloromethane.
g. Repeat steps d-e 3X more. (**Total of 4 washes**)
h. Discard combined washes in waste.
i. Rinse off yellow cap and larger top snap cap with Acetone (reduces contamination).
j. Dry yellow cap and top snap cap with paper towel.

10. <u>Kaiser Test (ninhydrin) – Used to verify amines on column have reacted with activated AA. (VERY IMPORTANT STEP)</u>

(Reference**:** Wellings, D. A.; Atherton, E. "Methods in Enzymology Volume 289: Solid-Phase Peptide Synthesis"Ed. Fields, G. B. Academic Press, San Diego, 1997, p. 54.)

Use self-made reagents (See below) (or Anaspec Ninhydrin Test Kit (#AS-25241))

Self-Made Kaiser Test Solutions (Save for future reactions)

Reagent A:

a. Dissolve 16.5 mg of KCN in 25 mL of distilled water.
b. Dilute 1.0 mL of above solution with 49 mL of pyridine (freshly distilled from ninhydrin).
c. Pour it into a small reagent bottle and label it "A".

Reagent B:

a. Dissolve 1.0 g of ninhydrin in 20 mL of n-butanol.
b. Pour into a small reagent bottle and label it as "B".

Reagent C:

a. Dissolve 40 g of phenol in 20 mL of n-butanol.
b. Pour it into a small reagent bottle and label it "C".

Kaiser Test Procedure

1. Using small metal spatula, dip the tip of the spatula into the column and gently remove approximately 10-15 of the resin beads and put in a microcentrifuge tube and label it "**S1**". (Knock spatula on sides of tube to dislodge the beads from the spatula) (if doing more than one sample, label w/ S2, S3, S4, etc.)
2. Label another empty microcetrifuge tube designated "**R**" (reference) (note only need to do this the first time so you can see color- should be no blue color in this tube)
3. **<u>To each tube add:</u>**
   25 µL of Reagent A
   25 µL of Reagent B
   25 µL of Reagent C
4. Heat both the tubes at 80°C for 5 minutes.

5. Compare the color with reference.
6. **RECORD** the result on the Peptide synthesis sheet. (**GOOD or BAD**)

Interpretation of Results (4 Possible Results)

-Colorless or very faint blue color: complete coupling, proceed with synthesis (**Best Result**).
-Dark blue solution but beads are colorless: nearly complete coupling, extend coupling or cap unreacted chains.
-Solution is light blue but beads are dark blue: coupling incomplete, recouple.
-Solution is intense blue and all beads are blue: failed coupling, check amino acid, reagents, then recouple.

11. **Starting with STEP #4, Repeat all steps except step 5 (#4, #6-#11 for each new amino acid to be added**
    - **-Deprotect**
    - **-Spectral analysis of FMOC released**
    - **-Wash Column**
    - **-Activate next amino acid**
    - **-Couple AA to column**
    - **-Wash Column**
    - **-Kaiser Test**

FINAL STEPS

12. **Once peptide is complete**, place column still containing the beads, in vacuum dessicator chamber overnight to dry.
13. **Pre-weigh** a 1 dram glass vial w/ cap. Label the vial with the following information:
    a. Weight of Empty vial with cap.
    b. AA sequence (N-term on left to C-term on Right with single letter abbreviations)
    c. Date
    d. Your name
14. Scrape all of the dried beads into the vial and get a final mass on the beads linked to final peptide (i.e. Vial with cap and the beads), **Put this on your label.**
15. **RECORD** this final mass on the Peptide synthesis sheet.
16. Subtract these values to get amount of peptide **(RECORD on Peptide SHEET)**
17. Use a piece of parafilm to seal the vial cap and place in the freezer (RM308) for future use.

General NOTES:

After approximately 3 Days, should be able to make 10mer amino acid

(Can leave as free amine or cap with acetyl group using acetic anhydride)

Cleavage Procedure (Check with DR. Werner to see scale fo Cleavage before proceeding)

1. Remove the peptide vial from the freezer, allow to stand approx. 30 min to equilibrate to room temperature before opening. (Peptides are hydroscopic)
2. Remove the parafilim and weigh out an appropriate amount of peptide linked beads into a microcentrifuge tube or small glass vial.

3. Make up a stock solution of 95% Trifluoroacetic acid (TFA) and 5% H_2O in a 15 mL scintillation vial.
    a. Add 9.5 mL TFA and 0.5 mL H_2O to 15 mL scintillation vial. (CAREFUL: TFA is quite acidic and may fume.) **SAVE FOR FUTURE REACTIONS.**
4. To cleave the peptide from the beads leaving a C-terminal 1^o amine, use a 40:1 ratio of TFA/H_2O volume to bead weight. (Example: 2.5 mg beads use 100 µL 95:5 TFA:H_2O ; 125 mg beads use 5.0 mL 95:5 TFA:H_2O)
5. **Carbocation Scavenger Cocktail.** Cleavage is often done with a cocktail of ethanedithiol (EDT) and triisopropylsilane (TIS). This reacts with released carbocations from deprotection steps and also helps protect sulfur containing amino acids (Cys, Met)
6. Place sealed reaction vessel on vortexer and let cleavage reaction proceed for 3 hours. This cleavage will not only cleave the peptide from the resin but also deprotect acid sensitive protecting groups.
7. Transfer the liquid to a syringe fitted with a 2 µM filter, push liquid into a clean lyophilization vessel to remove any resin beads.
8. Freeze liquid with dry ice/acetone and lyolphilize overnight.

Peptide Analysis

HPLC analysis using an analytical HPLC column
ESI mass spec analysis to get final mass of peptide
Lyolophize final cleaved peptide, store in sealed vial in freezer or desiccator.
NMR analysis of final peptide desirable.

Alternate Resins/Reagents

FMOC-Phe-Rink Amide MBHA resin (#20941) Anaspec? Pre-attached phenylalanine
HBTU
Simply agitate activation reaction for 1 min with vortexer

Manual Solid Phase Peptide Synthesis Sheet

***Sequence to be Synthesized:** _____
(N-term → C-Term, using single letter AA abbreviations)
(**NOTE**: 1st AA used in synthesis is actually C-terminal AA)

***Name of Researcher**:_____

***Date Synthesis was started:**_____

Record the following information about reagents used:
HATU Vendor:_____ Lot #:_____ Date Bottle Opened:_____
Amino Acid ID VendorPart # Lot # Date Bottle Opened

Mass of Starting Rink Amide Resin (mg):_____
ABS_{300} of 1st deprotection eluate:_____
Moles of "Free Amine" on Resin after step 5H:_____(Defines 1 eq.)

Initial Set-up

Reagent	**Mol. Wt. (g/mol)**	**# mol. Equiv.**	**Mol. Req.**	**Mass To weight out**	**Desired Conc. in NMP**	**Volume of NMP to add**
Amines on Resin	na	1 eq	na	na	na	na
HATU	380.2	2.85 eq			0.2M	
1st FMOC prot. AA (C-Term. AA)	**Look up**	3.0 eq			0.2M	
DIEA	129.25 d=0.742 g/mL	5.7 eq		na	2.0M stock solution from above	

First Kaiser Test Result:_____(Must be good to Proceed)

Fill in the following table for **each** amino acid used. Repeat scale calculations after **each** UV spectral analysis of FMOC released as in Step 5 and 7 of the procedure.

AA# from C-Term.	AA Used	µmol FMOC from UV	Mass HATU (mg)	Vol. NMP (µL)	Mass FMOC AA (mg)	Vol. NMP (µL)	Vol. 2M DIEA (µL)	Kaiser Good or Bad	Notes
2									
3									
4									
5									
6									
7									
8									
9									
10									
11									
12									
13									
14									
15									
16									
17									
18									
19									
20									

***Date Synthesis Completed:**_____

FINAL MASS

Weight of Vial and cap EMPTY:_____

Weight of Vial and cap with Peptide linked Beads:_____

Weight of Peptide (take difference of above two values:_____

Location of Beads linked to Peptide:_____(Freezer in 308 / Desiccator)

Cleavage Record:

Date Weight of Beads Used for Cleavage Weight of Final Dried Peptide

_____

_____

_____

_____

Analysis Notes:

HPLC:

MS:

NMR: