Note: this protocol is optimized for the Breeze software and for purifying peptides with a high concentration >30 mg/ml
To purify peptides with high performance liquid chromatography. Samples can be vortexed or sonicated if the peptide does not go into solution at a 30mg/ml concentration.

Objective
Peptides synthesized need to be purified with reversed-phase HPLC for quality control before use with cell culture materials. This procedure applies to the cleavage of a solid phase peptide synthesis of amino acid sequences in a rink amide resin. After peptides are validated via MALDI-TOF an analytical run on HPLC is used to determine purity or need of purification. If necessary, a purification is used to get the peptide sample to >95% purity.

MATERIALS
Trifluoroacetic acid, Optima (LC/MS grade) (Acros Organics cat. No. A116-2AMP)
HPLC grade Water (Sigma-Aldrich cat. No. 270733-4L)
Acetonitrile HPLC grade (Fisher Scientific cat. No. A998SK-4)
Methanol Optima (HPLC grade) (Fisher Scientific cat. No. A454-4)
20 um syringe filters
20 mL syringe (smaller volume)
1 mL glass syringe

SAFETY AND QUALITY CONTROL
Handling Acetonitrile:

1. Use in a well ventilated chemical hood. Wear safety glasses, gloves, and a lab coat. Store in a cool, dry area.
2. In case of eye contact: Check for and remove any contact lenses. Immediately flush eyes with running water for at least 15 minutes, keeping eyelids open. Do not use an eye ointment. Seek medical attention.
3. In case of skin contact: Wash the contaminated skin with running water for 15 minutes use an emollient. Cold water may be used. Wash exposed clothing before reuse.
4. In case of inhalation: Allow the victim to rest in a well-ventilated area. If the victim is not breathing, perform mouth-to-mouth resuscitation.
5. In case of ingestion: Do not induce vomiting. Loosen tight clothing. Seek immediate medical attention.

Handling Trifluoroacetic acid:

6. Use in a well ventilated chemical hood. Wear safety glasses, industrial grade gloves, and a lab coat.
7. Store in a dry area

8. In case of eye contact: Check for and remove any contact lenses. Immediately flush eyes with running water for at least 15 minutes, keeping eyelids open. Do not use an eye ointment. Seek medical attention.
9. In case of skin contact: Remove the contaminated clothes as quickly as possible, protecting your own hands and body. Place the victim under a deluge shower. It was exposed to victim's skin, such as the hands: Wash the contaminated skin with running water and non-abrasive soap. Cold water may be used. If irritation persists, seek medical attention. Wash contaminated clothing before reusing. For serious skin contact: Wash with a disinfectant soap and cover the contaminated skin with an anti-bacterial cream. Seek medical attention.
10. In case of inhalation: Allow the victim to rest in a well-ventilated area. Seek immediate medical attention. For serious Inhalation: Evacuate the victim to a safe area as soon as possible. Loosen tight clothing. If breathing is difficult, administer oxygen. If the victim is not breathing, perform mouth-to-mouth resuscitation.
11. In case of ingestion: Do not induce vomiting. Loosen tight clothing. If the victim is not breathing, perform mouth-to-mouth resuscitation. Seek immediate medical attention.

ANALYTICAL PROCEDURE

1. Two different buffer solutions must be prepared to run reverse phase high-performance liquid chromatography.
    a. Buffer A consists of water with 0.1% trifluoroacetic acid (1000 mL bottle)
    b. Buffer B consists of 0.1% trifluoroacetic acid in acetonitrile. (1000 mL bottle)
    c. Make fresh solution to ensure no impurities.
    d. Note: use only glassware to transfer PFA you don't want to include plastic in your sample
2. Prepare a 0.5mg/mL of desired peptide in Buffer A.
3. Filter peptide solution using a 0.20µm filter. (at least 1mL of peptide sample)
4.
5. Place reagents. Connected to the HPLC instrument are two lines labeled, A and B. These lines need to be inserted into their respective buffers and sealed with parafilm.
    a. Put the tubes in their respective buffers before opening the software because sometimes the pumps turn on automatically when the equipment is turned ON or when the software is booted.
6. Proceed to turn ON HPLC instrument. There are 4 switches that need to be turned ON for the instrument to work: 1) the computer, 2) the detector, 3) the pumps, and 4) the heater.
7. Wait for the UV detector to complete booting up. When complete, proceed to open the Breeze software located in the computer desktop.
8. After the software is loaded, press the "purge pump" button.
    a. The software will have an on-screen dialogue with instructions. These are described below.
    b. The first step is to prime each of the pumps to remove all of the air from the system. This is done by inserting a 20mL syringe into the valve below

the pump, opening the valve, and pulling in approximately 20mL of air/buffer. *Do this slow and steady*. The valve is then closed and the syringe is removed. Dispose of the solution in the waste container. It is critical to *insert the syringe first before opening the valve* as well as *closing the valve before removing the syringe* to ensure no air enters the system.

   c. Repeat point *b* for both of the pumps.
   d. For the purging of the pumps, the lever beneath pump A is switched to the right and the system purges at a set rate of 5 mL/minute for 3 minutes to flush the entire C18 column. *Failure to do this will result in flooding of the instrument.*

9. Press the “Equilibrate” button on the software and select the method “Lauren2”.
10. The system is left until the graph on the screen levels off.
    a. It takes >30 minutes to equilibrate.
    b. A dialogue box may pop up on the screen and the start menu may open. Press the “switch to” button on the dialogue box multiple times in a slow manner until it goes away.
11. The system reaches equilibrium when the graph illustrates a plateau. Press the “auto zero” on the HPLC instrument’s detector.
12. Turn down the instruments flow by 0.5 mL/min increments until flow reaches zero. Do this using the onscreen slider and pressing go after each adjustment.
13. Before injecting the sample into the HPLC instrument, the syringe needs to be cleaned by drawing and purging water, acetonitrile, and water into and out of the syringe in that respective order.
14. Draw 0.5mL of the sample into the syringe and keep ready for injection in the load position.
15. Press the “Make Single Injection” button.
16. Use the “Lauren2” method, set the injection to 500. The method time is set to 121 minutes to make sure the elution is slow and there is enough peak separation in case of impurities.
17. Press “inject” on the dialogue box. Follow the instructions on the on screen pop up. Move insertion switch to the open position, load your peptide sample and turn the switch below the injection hole to “Inject”. Wait 1 minute for the sample to load through the instrument and turn the switch back to “Load” after the injection has finished.
18. Take the syringe out and clean it again using the same water, acetonitrile, water process described earlier. (optional at the end of the run)
19. After the sample is finished, pumps A and B need to be primed and purged again, however this time with only acetonitrile.
20. To do this, make sure the pumps are not running. Insert all the lines (A and B) into the acetonitrile container and the top of the container must be sealed with parafilm.
21. Press the “Purge Pumps” button on the software and prime/purge the pumps using a purge setting of 50:50 Pump A:Pump B at 5mL/minute for 3 minutes to purge the entire system.
22. Analyze results to assess if purification is needed for the peptide.

23. Refer to special notes.

PURIFICATION PROCEDURE

1. Now that you have determined your peptide needs to be purified follow the purification procedure.
2. Make a method for the sample you are running. This is done using the information gained in the analytical run.
    a. The analytical run begins with a 10-minute start up process with a steady flow of 95% buffer A: 5% buffer B.
    b. From minutes 10 to 100 the flowrate is programmed to have a linear gradient from 95% buffer A: 5% buffer B to 5% buffer A: 95% buffer B
        i. $[A\%] = -t + 105$ (for $10 \leq t \leq 100$)
    c. Using the above equation, the initial and final percentage of A at any time can be determined. This is where the peptide elutes on the chromatograph made in the analytical run. Since the percentages of buffer A and buffer B together add to 100%, the respective buffer B concentration percentages can be determined.
    d. Once you determine the initial and final concentration at which the peptide eluted, make a new method using the Breeze software using the example below:

State	Time (min)	Flowrate (mL/min)	% Buffer A	% Buffer B	Curve
0	---	2.5	95	5	---
1	10	2.5	95	5	6
2	16	2.5	% initial	-%initial	6
3	22	2.5	% initial	-%initial	6
4	82	2.5	%final	-%final	6
5	88	2.5	5	95	6
6	100	2.5	5	95	6
7	102	2.5	95	5	6
8	112	2.5	95	5	6
9	114	0	95	5	6

    e. State 0-1: The method initially begins with a 10-minute start-up phase for the HPLC allowing the column to reach a flowrate of 2.5 mL/min and a 95:5 ratio of buffer A to buffer B.
    f. State 1: Since the column is 15 mL in volume and the flowrate rate throughout the process is 2.5 mL/min, 6 minutes is required to flush the column.
        i. The curve is always set to 6 to indicate linear change in flowrate in the program.
    h. State 2: The program is brought to the first concentration.
    i. State 3: The column is then flushed at these concentrations to ensure the entire column is at these concentrations.
    j. State 3 to 4: The flowrates are gradually, linearly changed to their final

elution concentrations over 1 hour.

k. State 5: The concentrations are then altered to 5% buffer A: 95% buffer B (state 4 to 5) over 6 minutes to flush the column to the final concentrations ending the elution. The column is then flushed at this concentration to ensure the sample is out of the column.
l. State 6-7: The concentrations are then varied linearly back to 95% buffer A: 5% buffer B and the column is flushed at this concentration (state 8).
m. The chromatograph is finally stopped at state 9 as the flowrate is brought down from 2.5 mL/min to 0 mL/min

3. Now prepare the peptide sample at the highest concentration possible in Buffer A. A starting concentration of 30 mg/mL is used. To help with dissolving follow either option:
    a. Dampen sample with 1-2 drops of acetonitrile and then dissolve in buffer A.
    b. Vortex sample.
4. The peptide solution is passed through a 0.20μm filter.
5. The HPLC is equilibrated using the Lauren2 method and is then zeroed (see above steps 1-10.
6. Press the “Make Single Injection” button.
7. Use the method you have created to purify the sample.
8. Press “inject” on the dialogue box, inject the sample into the HPLC instrument, and turn the switch below the injection hole to “Inject”.
9. Wait 1 minute for the sample to load through the instrument and turn the switch back to “Load” after the injection has finished.
10. Take the syringe out and clean it again using the same water, acetonitrile, water process described earlier.
11. During purification, the peptide sample elutes out of the HPLC. The elution line is connected to the waste container and can be removed to collect the sample.
    a. When a peak occurs on the chromatograph, detach the elution line from the waste container and put into a collection vial to collect the sample. (e.g. 20 mL glass vials)
    b. Each peak should be collected with a single vial labeled with the time of elution and the sample number to ensure all components were separated from the sample mixture.
    c. After the peak occurs on the screen, wait about 10 seconds before collection due to sample delay time.
    d. The largest peak is considered the peptide, but this should be validated via MALDI.
12. The purified sample is frozen (-80C) and then lyophilized for long-term storage. (Refer to lyophilization protocol. Training is required.)
13. After the sample is finished, pumps A and B need to be primed and purged again, however this time with only acetonitrile.
14. Make sure the pumps are not running. Insert all the tubes into the acetonitrile container and the top of the container must be sealed with parafilm.
15. Press the “Purge Pumps” button on the software and prime/purge the pumps using a purge setting of 50:50 Pump A:Pump B at 5mL/minute for 3 minutes to purge the

entire system.

SPECIAL NOTES

1. Waste bottle needs to be placed back into the waste area after you are done using the instrument.
2. %peptide purity = [(area UV 220nm DP)/(area UV 220 nm all peaks)]*100
    a. where:
    b. *area UV 220 nm DP:* is the area of the peak of the desired peptide in HPLC chromatograph monitored at 220nm
    c. *area UV 220 nm all peaks:* the sum of the areas of all peaks in the HPLC chromatogram including the area of the desired peptide.

CORRECTIVE ACTION

3. If the HPLC equipment will not be used in a considerable period of time store the column in methanol.
4. If the baseline is not horizontal or there is too much noise purge the column first with methanol and then with acetonitrile. Finally equilibrate the system following steps 1-10 of analytical procedure.
5. Make sure the waste container is not full or close to being full before proceeding with the run.

REFERENCES

N/A

APPENDIX

Annual review by: Sualyneth Galarza Date Reviewed: January 15, 2018