Bio-MS Research Core Facility

****

**Before you start:**

This method is suitable for the preparation of a wide variety of sample types for LC-MS analysis. It is assumed that you have already had a discussion about your work before attempting the steps described here. If not, contact us at bioms@manchester.ac.uk.

This protocol uses a number of chemicals and a risk assessment should be performed before starting.

We have COSHH forms in a red folder in the lab (above LDA1), where you can check the single substance risk assessment forms for each of the chemicals used in this protocol.

None of the chemicals used in this workflow are CMRs (carcinogen, mutagens, or reproductive toxins).

It is recommended that PPE be worn at all stages of the workflow, and remove gloves when using laptop keyboards for the direct detect and LE220+.

Above all, if you have any questions about H&S, or feel unsafe about any of the procedures– please ask and we will do our best to answer

Bio-MS Research Core Facility

**Title: Researcher led sample preparation for LC-MS using the BioMS research core facility**

**Before you start:**

This method is suitable for the preparation of a wide variety of sample types for LC-MS analysis. It is assumed that you have already had a discussion about your work before attempting the steps described here. If not, contact us at bioms@manchester.ac.uk. Afterwards, to get you started, we will provide you with Covaris lysis tubes for each sample that you wish to process, at cost.

**Part 01 – Sample lysis and protein extraction using the Covaris LE220+**

*1.1 Initial assumptions and preparation*

* Allow approximately 30 minutes for sample lysis and protein extraction.
* You may pre-book the use of the LE220+ machine beforehand on PPMS.
* If it is your first time using the LE220+, let us know at bioms@manchester.ac.uk, you will be provided with training, in which you will be provided with login rights to the machine and some basic processing methods to get you started.
* You have cells or tissue in Covaris tubes, on ice.

*1.2: Initial preparation*

**Before you begin:**

Log onto the LE220+ and check that the “run” box in the top right hand corner is green. If not, it may be that the system is not at temperature. Click the “System status” box on the right hand side – if a red “X” is next to temperature, then you must wait until the system reaches the temperature that you have set the system to in your method before you begin. The default temperature for the system is 10°C.

**Locate the following buffers, consumables, and reagents:**

|  |  |
| --- | --- |
| Location | Buffer/reagent |
| Fridge 02 | * N/A
 |
| Bench | * 2x stock of S-Trap lysis buffer {10% SDS, 100 mM triethylammonium bicarbonate (TEAB) pH 7.5}
* Eppendorf tubes, 0.5 or 1.5 mL volumes depending on sample volume.
* LC-MS grade water
 |
| Freezer | * N/A
 |

**Identify the following equipment that you will use:**

* 20ul pipette, 200uL pipette, 1ml pipette and pipette tips

# Stainless steel rack for LE220+ processing (500282 rack for 520045 tubes, 500452 rack for 520185 tubes) – Ask a member of BioMS staff for it.

# Spare Covaris tubes for making up the rows in the rack to either 8 per row for the 500282 rack, or 4 for the 520185 rack, check next to LE220+ machine.

*1.3: Preparing samples and LE220+*

1. You will need to dilute the S-Trap lysis buffer to a working stock concentration. Using the 1mL pipette, add 500 uL of 2x stock of S-Trap lysis buffer to 500 uL of LCMS grade water. This is the working stock concentration (5% SDS, 50 mM TEAB pH 7.5). If you know that your smaples are high in disulphide bonds, you may add 5 mM of dithiothreitol (DTT) at this stage. Refer to the section on “reduction and alkylation” for instructions on how to prepare DTT.
2. Add S-Trap lysis buffer (5% SDS with 50 mM TEAB pH 7.5). Do not freeze your samples once they contain buffer, it will cause the glass tube to crack.
	1. If using the 520045 tubes, add approximately 100 uL to 130 uL of buffer, the final volume will depend on the amount of sample already present in the tube.
	2. If using the 520185 tubes, add up to 500 uL of buffer, again this is dependent on the amount of sample that you wish to process.
3. Add the Covaris tubes to the respective stainless steel rack – According to Covaris, all spaces in the row must be filled, there are spare tubes beside the machine for this purpose. Tubes are processed in rows on the width of the plate.
4. Clamp the lid on the rack finger-tight, and keep the samples in the rack on ice until you are ready for processing.

*1.4: Sonolab 8.2 software*

1. On initial training, we will provide you with a general method that should be sufficient to lyse your sample. At the end of the process, if you feel that your sample would benefit from more treatment, simply run the program again.
	1. How will you know? With cell suspensions, the sample will turn from opaque to transparent, a sign that the cells have lysed.
	2. With tissue, after a quick centrifuge spin, you will see that your pellet is less than before.
2. Sonolab will automatically select the last method run by the user. To select an alternative method, use the drop-down menu to load an alternative method, it is located on the HOME tab, in the top left corner.
3. To specify the rows of the plate that you wish to process, click “edit”
4. This will open up the edit parameters page. If you have two rows of tubes to process, click on the process in the left hand side. This will allow the rows on the plate display on the right hand side of the page to be selected. Click on the two rows that you wish to process. When the LE220+ begins, it will process one row, then the next, in that order. The total processing time for what you specify in this page is displayed in the bottom right hand corner. Click save and return to the main menu.
5. The Run method will remain grey and inactive until a method is selected and all necessary conditions are met. On the right hand side, in the “Home” tab, take a look at the “System status” tab – if it is shaded red and contains a red X, then a starting parameter(s) has not been achieved – click on the button to see the issue. Usually it is because the set temperature has not been reached. By default the LE220+ is set to 10°C. If your method is set to 6°C, simply wait until the temperature has been reached.

#

*1.5: Running your method*

1. Click on the “Motion Control” box, and click on “load”, the plate holder will move to the front of the LE220+ for plate loading.
2. Open the door of the LE220+ by simultaneously pressing the green door lock button while gently pulling the handle of the safety enclosure Perspex door.
3. Load the plate with the orientation that you specified in the method.
4. When the “Run” button is green, the selected method may be started. Click the “Run” button. As the method runs, the graphic for the row under treatment flashes green, and the actual average incident power and temperature data are displayed.
5. When the method has finished, the message “Method Complete” is displayed. Click “OK”, then press the green door lock button and open the safety enclosure door. Remove the treated sample plate and place on ice.
6. Label 0.5 mL or 1.5 mL tubes for each 520045 or 520185 tube that you are processing, and transfer the lysed sample extract to the labelled tube.
7. You may now freeze your samples at this stage for storage, or proceed to reduction and alkylation.
8. If finished, **logout** of the Sonolab software and **return the rack** to a member of BioMS staff.
9. IMPORTANT: The Sonolab software should be always left ON, as by shutting it down, the default settings are triggered and the day to day maintenance settings are lost. If the software needs to be re-booted, contact a member of the facility.

***Do not shut down the software!***

*Notes:*

Treatments are defined by the following parameters: (For an in-depth explanation of these parameters, go to covaris.com)

* Treatment time.
* Cycles per Burst – the number of acoustic oscillations contained in each burst. The illustration below shows five cycles in each burst.
* Duty Factor – the percentage of active burst time in the acoustic treatment. The illustration shows a Duty Factor of 20%.
* Peak Incident Power – the power, in Watts, being emitted from the transducer during each burst.
* Average Incident Power - Peak Incident Power multiplied by the Duty Factor.



Bio-MS Research Core Facility

**Part 2: Reduction and alkylation using dithiothreitol and iodoacetamide**

**Before you start:**

* Reduction and alkylation of cysteine bonds is a prerequisite for LC-MS sample preparation for two reasons:
	+ Cysteine bonds are part of the proteins secondary structure. By reducing (breaking) them, it allows better access of trypsin or other digestion enzymes for the complete conversion of protein to peptide.
	+ It is not straightforward to identify cysteine containing peptides using LC-MS, because they usually are two peptides, linked by at least one cysteine bond. This will not be matched when the data is searched against the database, and will limit protein sequence coverage in the results.
* Dithiothreitol (DTT) is used to reduce the cysteine bonds present in the protein. After a short incubation, iodoacetamide (IAM) is added to modify the free cysteines. After another short incubation, DTT is added again to quench any free IAM.

*2.1 Initial assumptions and preparation*

* Allow approximately 90 minutes reduction and alkylation.
* You have protein lysates in Eppendorf tubes in a known volume of S-Trap lysis buffer.

*2.2: Initial preparation*

**Before you begin:**

Locate the Eppendorf Thermomixer, and attach the appropriate heating block depending on whether your samples are in 0.5 mL or 1.5 mL tubes. Set the temperature to 60°C and a speed of 500 RPM.

**Locate the following buffers, consumables, and reagents:**

|  |  |
| --- | --- |
| Location | Buffer/reagent |
| Fridge 02 | * DTT (PN# BP172-5, Fisher) pre-weighed aliquots – pink box – top shelf
* IAM (PN# I1149, Sigma Aldrich) pre-weighed aliquots – pink box – top shelf
 |
| Bench | * Eppendorf tubes, 1.5 mL volumes depending on sample volume.
* LC-MS grade water
* tinfoil
 |
| Freezer | * N/A
 |

**Identify the following equipment that you will use:**

* 20ul pipette, 200uL pipette, 1ml pipette and pipette tips.
* Kern fine balance

*2.3: Reduction and alkylation*

1. Remove the DTT and IAM aliquots from fridge 2. They are pre-weighed in 2 mL Eppendorf tubes. Take one of each for your preparation.
2. To make a 100 mM solution of DTT and IAM, add the volume of water indicated on the box from which you took the pre-weighed aliquot.
3. You will need to know the volume of the protein lysate sample that you wish to alkylate. If proceeding from LE220+ sample processing, this will be approximately 130 uL for the smaller tubes, and 500 uL for the larger tubes.
4. Reduce your protein sample. A final concentration (Fc) of 5 mM of DTT is used for reducing proteins. If the starting concentration (Sc) of the stock DTT is 100 mM, and the volume of sample (Fv) to be reduced is 130 uL, then use the following calculation to work out how much of the stock DTT (Sv) to add = (Fc \* Fv) / Sc. So for 130 uL of lysate, add (5 \* 130) / 100, or 6.5 uL of stock 100 mM DTT. For the 500 uL lysate, add 25 uL of the stock 100 mM DTT.
5. Place the tubes on the Eppendorf Thermomixer and heat for 10 minutes at 60°C. This reduces the cysteine bonds.
6. Alkylate your protein sample. Remove the protein samples from the Thermomixer, and allow to cool to room temperature. Add IAM. It is important to add at least three times the amount of IAM to DTT. So alkylate with 15 mM of IAM. For 130 uL of protein sample, add (15 \* 130) / 100 or 20 uL of IAM to the samples, and for 500 uL of sample, add (15 \* 500) / 100 or 75 uL of IAM to the samples.
7. Vortex mix the tubes briefly and place the protein sample tubes in the dark (a drawer) for 30 minutes.
8. After 30 minutes, add DTT to quench the alkylation reaction. Add the same amount of DTT again as to what you added in the reduction step. Vortex mix briefly.
9. Centrifuge your samples at 14,000 RCF for 10 minutes using the centrifuge in room B2075.
10. Remove the supernatant using a clean pipette tip to a labelled tube. This is the protein lysate.
11. You have now reduced and alkylated your protein lysate samples. You may now freeze the samples at this stage, or proceed to protein quantitation using the Millipore Direct Detect.

**Part 3: Measuring protein concentration using the Millipore Direct Detect.**



*3.1 Initial assumptions*

* Allow approximately 20 minutes for measuring the protein concentration.
* You will prepare a pool of your sample for protein measurement.
* You have cell or tissue lysates in S-Trap lysis buffer (5% SDS with 50 mM TEAB pH 7.5).
* Direct detect protein concentration measurement requires at least 6 uL of your sample.
* Protein lysates have been reduced and alkylated and clarified by centrifuging at 14,000 g for 10 minutes (see reduction and alkylation protocol).
* You will also need a blank sample to measure against. The blank is the buffer/solvent used to prepare the sample, but there is no protein in it. This should be 5% SDS with 50 mM TEAB pH 7.5 containing 10 mM DTT with 15 mM IAM.
* If you wish to do more than a single pooled measurement, you may buy a box of direct detect cards from us using PPMS. The cost of a card is approximately £1.60, a box is approximately £80 (price as of March 2019)

*3.2: Initial preparation*

**Before you begin:**

***Identify the following equipment that you will use:***

* 10uL pipette and pipette tips
* Millipore Direct detect sample cards (DDAC00010-GR)
* Millipore Direct detect machine

*3.3: Loading samples on card*

1. To measure the protein concentration of your lysate, place a direct detect card on a clean, dry surface. There are card holders available in the orange trays.
2. Label the bottom membrane on the card for blank measurement.
3. Label a clean 0.5 mL Eppendorf tube with “pool”. To it, add 2uL of each of your samples. When complete, vortex mix briefly.
4. Pipette 2 µL of blank into center of the membrane designated as the blank position.
5. Pipette 2 µL of your pooled sample to be analyzed into center of the three remaining spots. Take care not to touch the membrane with the pipette tip, as this might tear the membrane. Surface tension of the sample should be enough to pull it away from the tip onto the membrane.

*3.4: Software and measurement*

1. In the software, complete the fields as follows:
	1. User name should be preset
	2. Card name – today’s date, followed by your initials and the sample number
	3. Protein – use the drop down menu to select “proteins in S-Trap buffer.q3”
	4. Ensure that the box marked “dry sample card” is ticked.
	5. Do not tick the boxes marked “lipid”, “extended drying cycle”, “use previous blank”, or “modified mass unit”
	6. Give the 4 card positions a name, the position marked 1 in blue is the blank, while the other three should be in green, these are your three replicate measurements.
2. Insert the Assay-free card vertically into the instrument card holder with the instrument and card arrows aligned. Make sure that the writing side is facing the left. The instrument will move the card up and down and sound a tone. The green light illuminates to confirm proper insertion.
3. Click on the Measure Card button.
4. The sample concentrations will appear on the screen, along with the statistical analysis and spectrum plot.
5. After all four positions on the card have been read, the instrument will sound a tone. The card will rise to the initial insertion position. Remove the card and dispose of it. Previous measurements may be found under the history tab.

*Notes:*

The Direct detect is accurate for the measurement of protein lysates between 0.3 and 5 mg.mL-1. If you obtain a reading that is higher than 5 mg.mL-1, it will not be accurate, because the calibration curve is not linear above this concentration. To obtain an accurate measurement, dilute your sample 1 in 5 and 1 in 10 in SDS S-Trap lysis buffer containing 10 mM DTT with 15 mM IAM, and measure again in triplicate.

Bio-MS Research Core Facility

**Part 4: S-Trap™ 96-well plate digestion protocol**

**Initial assumptions**

* Allow 2.5 hours for S-Trap digestion
* You may perform S-Trap digestion using either the S-Trap plate, or S-Trap columns. Speak with a member of BioMS if you are unsure which option to use. The columns are best for loadings less than 50 ug of starting material, while the plates are best for approximately 100 ug. If you have a batch of n>10 samples, then you can use the plate.
* You have cell or tissue lysates in S-Trap lysis buffer (5% SDS with 50 mM TEAB pH 7.5).
* Protein lysates have been sheared of nucleic acid (e.g. from BioMS’s LE220+ AFA sonication system).
* Protein lysates have been reduced and alkylated and clarified by centrifuging at 14,000 g for 10 minutes (see reduction and alkylation protocol).
* The protein concentration of a pool of the protein lysates has been quantified and samples prepared to be 100 ug of protein in 50ul of 5% SDS (see notes).

**4.1: Initial preparation**

Before you begin:

**Locate the following buffers and reagents:**

|  |  |
| --- | --- |
| Location | Buffer/reagent |
| Fridge 02 | * Digestion buffer (50 mM triethylammonium bicarbonate)
 |
| Bench | * 12% phosphoric acid
* S-Trap binding buffer (90% aqueous methanol containing a final concentration of 100 mM TEAB, pH 7.1)
* 0.1% formic acid in water
* 0.1% formic acid in 30% acetonitrile
* MTBE / Methanol solution (10/3 (v/v))
 |
| Freezer 3 | * Aliquots of trypsin (10 uL at 2ug.uL-1)
 |

**Locate the following consumables:**

2x S-Trap plates (one to use and one as a balance in the centrifuge)

2x fresh collection plates

Tubes for (i) diluting samples and (ii) collecting unbound material (you can reuse sample tubes if desired)

You may need additional S-Trap binding buffer, there are aliquots of 5 mL of 100 mM TEAB, pH 7.1 stored in freezer 3, thaw and add 45 mL of methanol to make a final volume of 50 mL to use.

**Identify the following equipment that you will use:**

10ul or 20ul pipette, and a 1ml pipette

Thermo Megafuge 16 with plate rotor fitted

Vortex mixer

Eppendorf Thermomixer

Set the Eppendorf Thermomixer to 47°C, 60 minutes, and a speed of **0 RPM (i.e. no shaking)**.

**4.2: Loading samples**

1. To the SDS lysate, add 5ul of 12% aqueous phosphoric acid at 1:10 for a final concentration of 1.2% phosphoric acid. Vortex mix.
2. Add 350 μL of S-Trap binding buffer to the acidified lysis buffer. Mix.
3. Put the S-Trap plate on top of a clean 96 well plate, add the acidified SDS lysate into the plate. No plate preequilibration is necessary. Solution typically beings to drip through immediately.
4. Centrifuge the plate at 1,000 g for 2 min in the Megafuge 16 centrifuge.
5. Repeat the previous two steps until there all sample has been applied to the S-Trap plate***. Protein should be trapped within the protein-trapping matrix of the plate.***
6. Wash captured protein with **one** wash of 200 uL of MTBE solution, simply add 200 uL of the MTBE solution to the column, and spin at 1,000 g for 2 min. This will remove methanol insoluble biomolecules from the quartz filter.
7. Following this, perform **three** washes of 200 μL of S-Trap binding buffer, again, add 200 uL of the S-trap binding buffer, and centrifuge at 1,000 g for 2 min.

Note: if you wish, you may transfer the flow through and washes back into an Eppendorf sample tube after each centrifugation step, otherwise empty the collection tube so that the washes do not come in contact with the binding matrix. If discarding the washes then collect in a beaker and put in acetonitrile/solvent waste when finished.

**4.3: Digest proteins**

1. Move S-Trap digestion plate on top of a clean receiver plate.
2. Locate the trypsin aliquots. They are in the top shelf of freezer 3. Trypsin must be added to the protein at a ratio of 1:10 wt:wt (enzyme:protein). The frozen aliquots are at a volume of 10 uL containing 20 ug of trypsin (concentration of 2 ug.uL-1). Add 250 uL of digestion buffer to the aliquot. This gives a total volume of 260 uL, enough for 2 S-Trap digestions
3. Add 125 μL of digestion buffer containing protease into the top of the wells
4. Place cover over the stacked plates.
5. Incubate in the Thermomixer for 1 hr at 47°C for trypsin. ***Some dripping may occur during incubation; this is not of concern.* REMEMBER -** **DO NOT SHAKE.**

**4.4: Elute peptides**

1. Add 80 μL of digestion buffer to all wells of the S-Trap digestion plate. Centrifuge the plate at 1,000 g for 2 min or until all solution has passed through***. Do not centrifuge the plate prior to addition of 80 μL of digestion buffer used in this first elution.***
2. Add 80 μL of 0.1% aqueous formic acid to all wells of the S-Trap digestion plate and spin through at 1,000 g for 2 min.
3. Further elute peptides with 55 μL of 30% aqueous acetonitrile containing 0.1% formic acid and spin through at 1,000 g for 2 min. This elution assists in recovery of hydrophobic peptides. ***The final acetonitrile concentration will be around 5% v/v.***
4. Proceed to R3 desalting or store in a refridgerator overnight***.***

**Notes**

1. To create a 50ul sample at 1mg/ml protein calculate the amount of lysate required using the following calulation: amount lysate (ul) = 50ul/calculated pooled lysate concentration(mg/ml = ug/ul) and make up the volume to 50ul with 1x SDS solubilisation buffer, e.g. if pooled lysate was determined to have a concentration of 1.6 mg/ml then take 50/1.6 = 31ul of each sample and add 19ul of 1x SDS solubilisation buffer.
2. Protein quantification with direct detect on small amounts of sample is not that accurate so if your samples are about 1mg/ml or less (i.e. <1.3 mg/ml) then do not dilute the sample. The S-Trap plates can load much more protein if desired but you will need to increase the amount of trypsin used.

Bio-MS Research Core Facility

**Part 5: S-Trap™ column digestion protocol**

**Initial assumptions**

* Allow 2.5 hours for S-Trap digestion
* You may either do S-Trap digestion using either the S-Trap plate, or columns, speak with a member of BioMS if you are unsure which option to use. The columns are best for loadings less than 50 ug of starting material, while the plates are best for approximately 100 ug.
* You have cell or tissue lysates in 25 uL or 50 uL of S-Trap lysis buffer (5% SDS with 50 mM TEAB pH 7.5). If your lysates are lower than these two volumes, make up to either with S-Trap lysis buffer.
* Protein lysates have been sheared of nucleic acid (e.g. from BioMS’s LE220+ AFA sonication system).
* Protein lysates have been reduced and alkylated and clarified by centrifuging at 14,000 g for 10 minutes (see reduction and alkylation protocol).
* The protein concentration of a pool of the protein lysates has been quantified and samples prepared to be less than 4 ug.uL-1 of protein (Use the direct detect with the 5% SDS calibration file to check).

**5.1: Initial preparation**

Before you begin:

**Locate the following buffers and reagents:**

|  |  |
| --- | --- |
| Location | Buffer/reagent |
| Fridge 02 | * Digestion buffer (50 mM triethylammonium bicarbonate) (also known as TEAB)
 |
| Bench | * 12% phosphoric acid
* S-Trap binding buffer (90% aqueous methanol containing a final concentration of 100 mM TEAB, pH 7.1)
* 0.1% formic acid in water
* 0.1% formic acid in 30% acetonitrile
* MTBE / Methanol solution (10/3 (v/v))
 |
| Freezer 3 | * Aliquots of trypsin (10 uL at 2ug.uL-1)
 |

**Locate the following consumables:**

S-Trap columns for processing 1.5 mL (i), and 2 mL (ii) sample tubes for (i) diluting samples and (ii) collecting unbound material.

**Identify the following equipment that you will use:**

20uL, 200ul, and 1000 uL pipette

Eppendorf centrifuge 5430R

You may need additional S-Trap binding buffer, there are aliquots of 5 mL of 100 mM TEAB, pH 7.1 stored in freezer 3, thaw and add 45 mL of methanol to make a final volume of 50 mL to use.

 Vortex mixer

Eppendorf Thermomixer

Use the 1.5 mL adaptor for the Eppendorf Thermomixer, and set it to 47°C, 60 minutes, and a speed of **0 RPM (i.e. no shaking)**.

**5.2: Loading samples**

1. To the SDS lysate, add 2.5ul or 5uL of 12% aqueous phosphoric acid at 1:10 for a final concentration of 1.2% phosphoric acid. Vortex mix. Total volume is now either 27.5uL or 55 uL.
2. Add 165 μL or 330 uL of S-Trap binding buffer to the 27.5 uL or 55 uL volumes of acidified protein lysates respectively.Mix.Total volume is now 192.5 uL for the 25 uL starting volume, or 385 uL for the 50 uL starting volumes.
3. Place the S-Trap column on top of a 2 mL Eppendorf tube. This will collect the flow-through. Add enough of the acidified methanolic lysate to the S-Trap column. Do not add more sample than will fit the narrow “stem” of the S-Trap column. If the volume to be loaded is larger than will fit in the stem, then proceed to centrifugation (next step),
4. Centrifuge the column/tube combination at 4,000 RCF for 2 min in the Eppendorf 5430R centrifuge. ***Protein should be trapped within the protein-trapping matrix of the column.*** It is important not to let the liquid that passes through the S-Trap to come in contact with the protein-trapping matrix of the column.
5. Repeat the previous two steps if there is additional sample to be processed.
6. Wash captured protein with **one** wash of 150 uL of MTBE solution, simply add 150 uL of the MTBE solution to the column, and spin at 4,000 g for 2 min. This will remove methanol insoluble biomolecules from the quartz filter.
7. Following this, perform **four** washes of 150 μL of S-Trap binding buffer, again, add 150 uL of the S-trap binding buffer, and centrifuge at 4,000 g for 2 min.

Note: if you wish, you may transfer the flow through and washes back into an Eppendorf sample tube after each centrifugation step, otherwise empty the collection tube so that the washes do not come in contact with the binding matrix. If discarding the washes then collect in a beaker and put in acetonitrile/solvent waste when finished.

**5.3: Digest proteins**

1. Move S-Trap column to a clean digestion 1.5 mL Eppendorf tube.
2. Locate the trypsin aliquots. They are in the top shelf of freezer 3. Trypsin must be added to the protein at a ratio of 1:10 wt:wt (enzyme:protein). The frozen aliquots are at a volume of 10 uL containing 20 ug of trypsin (concentration of 2 ug.uL-1). If working from 50 ug of protein starting material, then add 75 uL of digestion buffer. This gives a total volume of 85 uL, enough for 4 S-Trap digestions. If your amount of protein starting material is 25 ug, make up the aliquot with 155 uL of digestion buffer, enough for 8 S-Trap column digestions. Add 20 μL of digestion buffer containing protease at 1:10 wt:wt into the top of the micro column. Use a gel loading tip (blue box) for this step. **IMPORTANT! – Ensure there is no air bubble between the digestion buffer and the column matrix.** The S-Trap binding matrix is hydrophillic and will absorb the digestion buffer when incubating.
3. Cap the S-Trap column to limit evaporative loss.
4. Incubate in the Eppendorf Thermomixer for 1 hr at 47°C for trypsin. ***Some dripping may occur during incubation; this is not of concern.* REMEMBER -** **DO NOT SHAKE.**

**5.4: Elute peptides**

1. Add 65 μL of digestion buffer to the S-Trap column. Centrifuge the column / tube at 4,000 g for 2 minutes, and collect.
2. Add 65 μL of 0.1% aqueous formic acid (FA) to the S-Trap column. Centrifuge the column / tube at 4,000 g for 2 minutes, and collect, this is now combined with the first elution through the centrifugation process.
3. Add 30 μL of 30% aqueous acetonitrile containing 0.1% formic acid. Centrifuge the column / tube at 4,000 g for 2 minutes, and collect .This elution assists in recovery of hydrophobic peptides. This is now combined with the the first and second elutions. The total volume will be approximately 200 uL.***The final acetonitrile concentration will be around 5% v/v.***
4. Proceed to R3 desalting or store in a refridgerator overnight***.***

Bio-MS Research Core Facility

**Title: 96-well plate R3 desalt and clean up protocol for mass spec analysis**

**Initial assumptions**

* Allow 60 minutes for R3 desalting, plus 90 minutes time spent drying down time
* You have digested samples that have no detergents in them (e.g. from S-Trap)
* Samples contain 5% or less organic solvent – dilute samples if necessary (see notes) {need some solvent to solubilise peptides}
* Sample volume is 50ul to 200ul. If less then dilute with 0.1% formic acid, if more then use multiple loading steps

**6.1: Initial preparation**

Before you begin:

**Locate the following buffers and reagents:**

|  |  |
| --- | --- |
| Location | Buffer/reagent |
| Bench | * 0.1% formic acid in water
* 50% acetonitrile
* 0.1% formic acid in 30% acetonitrile
* Oligo R3 beads in 50% acetonitrile
 |

**Locate the following consumables:**

2x Corning FiltrEX desalt filter plates (one to use and one as a balance in the centrifuge)

2x fresh ABgene storage/collection plates

Tubes for collecting unbound material (you can reuse sample tubes if desired)

Mass spec sample vials and caps (blue for samples, red for a pooled sample).

**Identify the following equipment that you will use:**

10ul or 20ul pipette

1ml pipette

Thermo Megafuge 16 with plate rotor fitted

Eppendorf Thermomixer

Vacuum centrifuge

**6.2: Preparing plates**

1. Add 10 µL of the prepared POROS R3 (from the settled beads) to the appropriate number of unused wells the filter plate. ***Ensure you know which ones you are using (e.g. mark the wells)***
2. Wash the filter plate and beads combination once with 200 uL of 50% acetonitrile and centrifuging at 200 g for 1 minute. ***Discard the into the temporary solvent waste beaker and transfer to solvent waste.***
3. Wash the plate twice with 200 uL of 0.1% formic acid in water. ***Dispose of waste into acetonitrile waste (lab).***

**6.3: Load sample and wash beads**

1. Add a maximum of 200 uL of the samples to the plate and beads. Incubate samples on the plate mixer for 5 minutes at 500 RPM **with no heating**.
2. Remove liquid by centrifugation at 200 g for 1 minute. ***Save flow through by transferring it to the original sample tube***
3. Repeat the previous two steps if there is additional sample to be processed.
4. Wash 1: Add 200 uL of 0.1 formic acid, mix for 2 min at 500 RPM, centrifuge at 200 g for 1 min. ***Combine wash with flow through sample to be safe – ensure you are using the correct solution for this step!***
5. Wash 2: Repeat the previous step

**6.4: Elute peptides**

1. Change the collection plate to one with unused wells.
2. Elution 1: Add 50 uL of 0.1% formic acid in 30% acetonitrile, mix for 2 min, 500 RPM, centrifuge at 200 g for 1 min.
3. Elution 2: Repeat the previous step.
4. The combined elution volumes should be approximately 100 uL. Transfer the combined elution to sample vials.
5. If you wish to prepare a pooled sample, you can do it at this step. See notes.
6. Dry samples to completeness in Heto vacuum centrifuge (see facility staff for how to use the vacuum centrifuge to dry peptide samples, or see the next section that details the procedure). Peptides may be stored in fridge or freezer when dried down.

Notes:

Pooled samples: Only do this if a member of staff has advised you to do so. To prepare the pooled sample(s), -for every 10 samples, remove 9 uL from each into another MS vial labelled “pool”. You will now have 10 samples with 90 uL of sample, and a pooled sample of 90 uL. We will use the pooled sample for QC (quality control). To make it easy to differentiate the “pooled” sample of peptides from the other peptide samples, use a red screw cap instead of a blue cap to close the vial. We will provide you with these caps.

****

**Title: Using the Thermo SPD1010 speed vacuum concentrator centrifuge**

**Initial assumptions**

* You have peptides in non-chlorinated solvent that you need to remove, and thereby concentrate and store your sample.
* The speed vacuum (speedvac) process works by drying the sample at the bottom of the vial recovery and storage. It takes place under vacuum conditions, which promotes solvent evaporation in the speedvac chamber. Centrifugation generates a centrifugal force to prevent bumping (bubbling), boiling, and physical loss of the sample. Samples are maintained in liquid state at sub-ambient temperature throughout the concentration process, preventing loss of activity or damage to heat sensitive substances. By applying a controlled vacuum the boiling point of the solvents can be lowered to the point that the liquid vaporizes with minimal heat which prevents oxidation of samples during the drying process.
* For 120 to 150 uL of peptides in 30% acetonitrile, allow 75 to 90 minutes to dry the peptides and evaporate the solvent.

**7.1: Initial preparation**

Before you begin:

**Locate the Thermo SPD1010 speedvac,** it is at corner of the sample prep lab in room B2075

**Locate the following consumables:**

* Vial caps with the white septa removed (contained in a blue box)

**We will ensure that there is sufficient Cryocool present before use**.

 A line on the wall of the stainless steel trap indicates the minimum appropriate level. Cryocool conducts heat away from the glass condensation flask, allowing vapors to condensate on the flask walls.

**Before you start: On the right hand side of the machine, there is a white rubber gasket -** It is important to ensure that the white flask insulating seal is over the glass flask to secure the flask in the cooling chamber.

**7.2: Speedvac drying.**

1. Turn the power switch located on the rear right hand side of the unit to the ON position. Wait 45 minutes before starting drying. This is to allow the system to come to temperature. The cover lock disengages, allowing the top cover on the left side of the unit to be opened.
2. After waiting, add the vials to the centrifuge by placing the neck of the vial in and under the hole, and secure into place by screwing a blue vial cap onto it. Do this for all of your samples, and balance them as you would for any centrifuge, across the spindle.
3. Close the lid, and switch on the vacuum pump on top of the cold trap.
4. Use a manual run for drying, this allows you to intermittently check the progress of the vacuum drying procedure. Using the SELECT button and the up/down keys, set the temperature to “NO” for no heat. Set the heat time to “CCC”. No run time is necessary since this is a manual run. There is no need to select a vacuum level.
5. Press the “MANUAL RUN” button. The cover locks and the rotor starts turning. The decimal point blinks. If the cover is not closed, the display will show “Lid” and the run will not start.
6. To end the manual run, press the “STOP” button. The display will show “END”, the valves will click, and the chamber will isolate from the vacuum pump allowing air into the chamber.
7. After the rotor stops spinning, the cover lock disengages and the display reverts to the last set paramaeters, and the unit will emit several audible beeps.
8. Open the cover and remove the samples.
9. When the lid click open, lift it and remove the vials, if dry, you can replace the speepvac cap with ordinary blue LCMS caps, located on the workbench in a square box.
10. If no one else has samples in the speedvac, and the plate speedvac is not in use, switch off the vacuum pump. It is not essential to switch off the vacuum readback.
11. Proceed to sample submission.

Bio-MS Research Core Facility

****

**6.1: Sample Submission**

1. Place the dried down peptides into the drawer in zone 1, either in a labelled bag (<20), or box if you are submitting a large batch of samples.
2. On the label, you must include PPMS order number (not project), number of samples, and any other important details, such as run length, sample manifest (i.e. which order you wish the data to be acquired in, if minimisation of carryover is important), and and whether the samples have been prepared in S-trap lysis buffer, or another buffer (e.g. urea, triton etc). Don’t forget to include your lab group/PI, as this is the folder within which your data files will be stored on the server.

**Notes**

 **Manifests:** Go to PPMS, and prepare a manifest relating to the samples, pay particular attention to the run order that you would like. Any analytical technique will suffer from a degree of sample carryover, LC-MS is no different. For example, if you have knockdown/knockout samples, it is recommended that you run these before your WT/control samples. Add in the position of the blanks/standards that you would like to see, we usually add these between every six 90-minute runs.