**Standard Operating Procedure: Tissue for LCMS by Prep by Zoom IEF**

This protocol accompanies the following videos:

[Tissue Prep for LCMS – Pt.1: Sonication](http://www.benchfly.com/video.php?video=115)

[Tissue Prep for LCMS – Pt.2: BCA Assay](http://www.benchfly.com/video.php?video=118)

[Tissue Prep for LCMS – Pt.3: Alkylation/Digestion](http://www.benchfly.com/video.php?video=129)

[Tissue Prep for LCMS – Pt.4: Desalting](http://www.benchfly.com/video.php?video=131)

[Tissue Prep for LCMS – Pt.5: ZOOM Isoelectric Focusing (IEF)](http://www.benchfly.com/video.php?video=116)

[Tissue Prep for LCMS – Pt.6: Cutting ZOOM Strips](http://www.benchfly.com/video.php?video=117)

[Tissue Prep for LCMS – Pt.7: Extracting Peptides from ZOOM Strip](http://www.benchfly.com/video.php?video=132)

[Tissue Prep for LCMS – Pt.8: Final Desalting](http://www.benchfly.com/video.php?video=133)

**1.0 Overview**

 1.1 This SOP is written to detail the steps taken to prepare a tissue sample for analysis by MS.

**2.0 Reagents and Materials**

* 1. AmBic (Ammonium Bicarbonate) Fisher Scientific - Cat#A643-500.
	2. TFE (2,2,2-Trifluoroethanol), 99.8% pure Acros -Cat#1397510000.
	3. TCEP-HCl Pierce Protein Research Products - Cat#20490.
	4. DTT (1,4-Dithiothreitol), No-Weigh DTT Fisher Scientific - Cat#PI-20291
	5. IAM (Iodoacetamide), 98% Sigma Aldrich Inc. - Cat#A3221-10vl
	6. Trypsin Gold, Mass Spectrometry Grade Promega - Cat#V5280.
	7. Formic Acid (FA), Suprapur, 98% EMD Chemicals - Cat#11670-1.
	8. Trifluoroacetic Acid (TFA), 99.0% assay Fisher Scientific - Cat#04902-100.
	9. Urea Fisher Scientific - Cat#BP169-500
	10. Acetonitrile (ACN), HPLC Grade Fisher Scientific - Cat#A998-1.
	11. Water, HPLC Grade, J.T. Baker VWR – Cat#JT9823-1

**3.0 Apparatus/Instrumentation**

* 1. SEP-Pak vac 1cc, 100mg, C18 Waters - Cat#023590.
	2. Oasis HLB Cartridge 1cc/10mg, 30µm Waters - Cat#186000383
	3. Oasis HLB Cartridge 3cc/60mg, 30µm Waters – Cat#WAT094226
	4. Oasis HLB 96-well plate, 30µm (10mg) Waters - Cat#186000128
	5. Oasis HLB 96-well plate, 30µm (5mg) Waters - Cat#186000309
	6. Oasis HLB 96-well plate HLB µElution Plate Waters - Cat#186001828BA

30µm

* 1. ZOOM IPGRunner Mini-Cell Invitrogen - Cat# ZM0001
	2. ZOOM IPGRunner Cassettes Invitrogen - Cat# ZM0003
	3. ZOOM Custom Order pH Strips (pH 3.5-4.7)
	4. Invetrogen ZOOM Dual Power Invitrogen - Cat# ZM10001

(100-120 VAC 47 – 60 Hz) œ

* 1. Nunc deep well plates, 2mL, sterile Nalge Nunc International - Cat#278743
	2. Falcon 96 well Microplates, clear, BD Biosciences - Cat#351172

flat-bottom with lid, sterile, polystyrene

* 1. Falcon Centrifuge Tubes, conical-bottom, BD Biosciences - Cat#352097

15mL, sterile, polypropylene

* 1. Vortex Mixer, Analog, 120V Fisher Scientific - Cat#02-215-365
	2. Balance, Ohaus Voyager Pro, Dual Range Scale Ohaus - Cat#VP214DCN
	3. Eppendorf Thermomixer Fisher Scientific - Cat#05-400-200

**4.0 Procedure**

Note: This procedure is separated into sections (by ------) based on what is typically done each day. The times given are estimated prep times needed to complete each step of the procedure.

Day 1 ~ 6 hours with digestion

4.1 Add 100µLof TFE and 100µL of 50mM Ambic, pH 8.0, to tissue in a 1.5mL micro-centrifuge tube. (Note: You may need to use more if needed, remember always use equal amounts.)

4.2 Using the microtip, sonicate (at setting 2) continuously for 20 seconds, then ice for at least 30 seconds, or until cool (Sample should not feel at all warm prior to sonication). Repeat sonication two more times, making sure to ice the samples in between sonications.

4.3 Incubate at 60°C for 60 minutes at 1000rpm on the Eppendorf Thermomixer.

(Note: Turn thermomixer on prior to incubating samples so that the temperature is 60°C at the beginning of the 60 minutes period.)

4.4 Using the microtip, sonicate (at setting 2) continuously for 20 seconds, then ice for at least 30 seconds. Repeat sonication two more times, being sure to ice the samples in between sonications.

4.5 Perform BCA analysis – following instructions in Pierce BCA kit.

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Note: You can store samples in the -80° freezer at this point.

* 1. At this point, you should set up your experiment. You know how much protein you have in each sample. First, you should aliquot out the desired amount of each sample needed and use the chart below to guide you on the amounts of TCEP/DTT and IAM for reduction and alkylation before the trypsin digestion.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Amount of Sample** | **TCEP** | **DTT** | **IAM** | **50mM AMBIC**  |
| 0-50ug (35uL total) | 20mM  | 50mM (5uL) | 100mM (5uL) | 200uL |
| 51-250ug (100uL total) | 20mM  | 50mM (20uL) | 100mM (20uL) | 360uL |
| 251-1500ug (200uL total) | 40mM  | 100mM (100uL) | 200mM (100uL) | 600uL |

* 1. Add TCEP/DTT solution and incubate at 60°C for at least 30 minutes at 1000rpm on the Eppendorf Thermomixer.

To make: 20mM TCEP/50mM DTT solution: 5.8mg TCEP and 7.7mg DTT (1 tube of No-weigh DTT)

 40mM TCEP/100mM DTT solution: 11.5mg TCEP and 15.4mg DTT (2 tubes of No-weigh DTT)

 (both made in 1mL 50mM AmBic, pH 8.0)

* 1. Cool samples to room temperature. Add IAM (made in HPLC grade water).

Incubate at room temperature in the dark for 20 minutes.

To make: 100mM IAM solution:

 200mM IAM solution:

* 1. Dilute sample with enough 50mM AmBic, pH 8.0 (making 10% TFE). Check the pH of this solution. Your pH should be 8 for adequate digestion.
	2. Add trypsin at a ratio of 1:50 (w:w) based on how much protein you have in each tube.
	3. Incubate overnight at 37°C.

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Day 2 ~ 30 minutes + Lyophilizer time

* 1. Freeze samples at -80°C. Turn on lyophilizer. Allow 30 minutes for the sample to reach -80°C and to allow the lyophilizer to reach the correct temperature and pressure). Put the samples in the lyophilizer and run until dry, usually overnight.

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Day 3 ~ 2 hours +Speed Vacuum dry time.

(Note: This desalting step can be done many ways. Please see chart for options according to sample amount.)

|  |  |
| --- | --- |
| **Desalting Product** | **Amount of Protein (ug)** |
| Oasis HLB 96-well plate HLB µElution Plate, 30µm |  |
| Oasis HLB 96-well plate, 30µm (5mg)  |  |
| Oasis HLB 96-well plate, 30µm (10mg)  |  |
| SEP-Pak vac 1cc, 100mg, C18 |  |
| Oasis HLB Cartridge 1cc/10mg, 30µm |  |
| Oasis HLB Cartridge 3cc/60mg, 30µm  |  |

* 1. Re-suspend samples in 1mL of HPLC grade water and vortex vigorously for one minute.

* 1. Spin down particular matter (quick spin).
	2. Desalt. Do not load more that 250ug per cartridge, you can overload the desalting filter.

4.15.1 1mL 100% ACN, charge by gravity flow. (Rinse solvent may be discarded)

4.15.2 2mL HPLC grade water, charge by gravity flow, if feasible. (Rinse solvent may be discarded)

4.15.3 Load 1mL sample. Elute at a rate of 0.2-0.5mL/min. Ideal elution time is about five minutes. Save eluent in vial/plate and store at -20°C labeled as sample name and LOAD.

* + 1. Wash with 1mL HPLC grade water. Elute at a rate (0.2-1.0mL/min). Ideal elution time is about five minutes. Save eluent in vial/plate and store at -20°C labeled as sample name and WASH.

* + 1. Elute with 1mL 80% ACN. Apply vacuum to recover as much eluent as possible. This elute in vial/plate and should be labeled as sample name and ELUTE.
	1. Dry ELUTE in Speed-Vac to dryness. This should only take a few hours during the day.

Note: Store dry samples in freezer overnight or until you can complete the next steps.

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Day 4 ~5 hours

* 1. Dependant on how much protein you want to run on a single Zoom strip, you should either:

Reconstitute your sample(s) in water and aliquot what you need to run, and then dry that down in the speed vac and then reconstitute in 155uL 6M Urea

(or)

Take the dried sample from 4.16 and directly reconstitute dry samples in 155uL 6M Urea. Vortex samples vigorously for one minute. Centrifuge as necessary.

(Note: If you are digesting large amounts of protein and only need aliquots for the next steps, DO NOT store samples in UREA. You should store the samples in water.)

 (Note: Make Urea fresh daily.)

 6M Urea = molecular weight (60.06g) x 6 = 360.36g/ 1L

 6M Urea = 0.72g/2mL

4.18 Load sample (155uL) into the sample loading wells located at the rounded edge of the ZOOM IPGRunner cassette. (You do not need to load the remaining wells if you are not planning on using them for rehydration…..leave the unused wells empty.)

4.19 Remove Zoom strip (pH 3.5-4.7) from card holding from the basic (-) end, flipping and making the strip gel side up.

 4.20 Using your fingers, gently slide the acidic (+) end of the strip into the sample well located on the curved

 side of the cassette. (To avoid air bubbles, slide the strip back and forth until all bubbles are gone.)

 4.21 Seal all sample wells with the purple sealing tape provided in the kit.

 4.22 Incubate at room temperature for at least 1 hour.

 4.23 Remove sealing tape and white sample loading device from cassette to expose adhesive.

* 1. Apply (600uL) DI water to 2 electrode wicks each.
	2. Place an electrode wick at both ends of the cassette over the adhesive, using the black alignment marks.

4.26 Slide the cassette into position into the ZOOM IPGRunner core rounded side up, and the wicks will be in contact with the electrodes of the core. (If only 1 cassette is being focused, use the buffer dam in the core, however, if 2 cassettes are being focused, the buffer dam is not needed)

4.27 Slide the apparatus with the core, cassette, and buffer dam into the mini-cell chamber core.

4.28 Pull the gel tension lever of the wedge toward the front of the mini cell until the lever tight.

4.29 Fill the **OUTER** chamber of the mini cell with 600mL DI H2O. **CAUTION: Do not pour any other liquid into the inner chamber of the mini cell.**

* 1. Place the ZOOM IPGRunner cell lid on the ZOOM core.

**CAUTION: Do not handle the lid with the electrode cords plugged into the power supply.**

* 1. With the power supply turned off, connect the electrode cords to the power supply.

Turn the power on and perform IEF with the following method: (it is preloaded on the power supply under method #1) 175V for 15 min

 175-2000V ramp for 45 min.

 2000V for 105 min.

Note: (Make sure the power supply is working before you leave the strips to focus. When the method starts, look to see if there are mAMPS, if not the strips are not focusing and the power supply needs troubleshooting.)

4.32 The run is 2 hours and 45 min. total. The power will automatically cut off. Turn the power off and disconnect the cables from the power supply. Remove the lid and pour off the water. Unlock the gel tension wedge and remove the ZOOM cassette.

4.33 Carefully peel off the clear film to get to the strips.

4.34 Cut each strip into 10 fractions and place gel side up in a 96 well plate.

 (Cut strips can be stored in the -80° freezer.)

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Day 5 ~2 hours + Speed Vacuum Dry Time

4.35 Extract peptides off strip with the following solutions and place each serial extract in 1.5mL micro tubes (Label 20 tubes accordingly, i.e. A1, A2,…A20):

* + 1. 200µL 0.1% FA. Incubate 15 minutes at room temperature. Pipette solution into appropriate tube (i.e. A1, A2,…A20).
		2. 200µL 50% ACN/0.1% FA. Incubate 15 minutes at room temperature. Pipette solution into appropriate tube (the respective fraction tube from the previous step).
		3. 200µL 100% ACN/0.1% FA. Incubate 15 minutes at room temperature. Pipette solution into appropriate tube (the respective fraction tube from the previous step).
	1. Dry samples in Speed-Vac ( 4-6 hours). Use the medium setting.

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Day 6 ~4 hours + Speed Vacuum Dry Time

 (Note: As noted before, with various amounts of protein, different products may be used.)

|  |  |
| --- | --- |
| **Desalting Product** | **Amount of Protein (ug)** |
| Oasis HLB 96-well plate HLB µElution Plate, 30µm | 60-400ug |
| Oasis HLB 96-well plate, 30µm (5mg)  | 150-1000ug |
| Oasis HLB 96-well plate, 30µm (10mg)  | 350-2000ug |

4.37 Re-suspend sample in 750uL 0.1% TFA.

4.38 Desalt using the Oasis HLB 96-well plate HLB µElution Plate, 30µm as follows:

 4.38.1 750uL ACN, charge slowly with gravity. (Rinse solvent may be discarded.)

 4.38.2 1400uL 0.1% TFA, charge slowly with gravity. (Rinse solvent may be discarded.)

 4.38.3 750uL Sample LOAD, try for a 3-5 minute elution time, or longer. (SAVE LOAD in 2mL plate)

 4.38.4 750uL 0.1% TFA, WASH, try for a 5 minute elution time, or longer. (SAVE WASH in 2mL plate)

 4.38.5 ELUTE as follows:

 4.38.5.1 200uL 30% ACN/0.1% TFA.

 4.38.5.2 200uL 70% ACN/0.1% TFA.

 4.38.5.3 200uL 100% ACN/0.1% TFA.

 4.39 Dry ELUTE in the Speed-Vac overnight.

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Day 7 ~1 hour

 4.30 Re-suspend samples in desired volume for protein load as described in the following table.

|  |  |
| --- | --- |
| **Protein Load** | **Reconstitution Amount of 0.1% Formic Acid (uL)** |
| 50-100ug total protein/strip | 25uL |
| 101-250ug total protein/strip | 100uL |

 4.40 Vortex on setting 4 for 15 minutes.

 4.41 Centrifuge samples in Speed- Vac for 10 seconds.

 4.42 Transfer samples to HPLC vials. Samples are ready for analysis.