STANDARD OPERATING PROCEDURE

Title: Robotic Assay Procedure for with ESI-MS Detection Using a KingFisher™ Flex Magnetic Particle Processor and Shimadzu LCMS 8050

SOP#: M-131

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Date Approved: 4-5-2018 **Date Modified:**

1. PURPOSE

This procedure will describe how to prepare antibodies / peptide antigen samples subjected to robotic characterization and purification with subsequent analysis by reverse phase LC and ESI mass spectrometry.

2. SCOPE

This procedure applies to all samples that are processed for analysis using the KingFisher™ Flex Magnetic Particle Processor and analyzed using the Shimadzu LCMS 8050. This procedure will describe sample preparation and instrument operation for the KingFisher™ Flex Magnetic Particle Processor and Shimadzu LCMS 8050.

3. RESPONSIBILITIES

It is the responsibility of the person(s) executing this procedure to be familiar with lab safety procedures and to have basic laboratory skills. It is the responsibility of the analyst to follow the procedure and document any deviations and all observations in the laboratory notebook.

4. EQUIPMENT

4.1. Shimadzu LCMS 8050

4.2. Mini Vortexer, VWR

4.3. Spectrafuge Mini Centrifuge, Labnet International Inc.

4.4. DynaMag™-96 Side Skirted Magnet (Cat. Number 12027)

4.5. KingFisher™ Flex Magnetic Particle Processor with 96 KF Head (Thermo Scientific)

4.6. Tip Comb for KingFisher™ Flex 96 KF Head (Cat. Number 97002524)

4.7. Eppendorf Centrifuge 5810R with plate rotor

4.8. Mettler AJ100 Analytical Balance

5. MATERIALS

5.1. DynaMag™-96 Bottom (Cat. Number 12332D)

5.2. KingFisher 96 KF microplate, 200µL (Cat. Number 97002540)

5.3. Thermo Scientific V-Bottomed 96-well Storage plate (Cat. Number AB-1058) (PCR plate)

5.4. Weighing Paper, 3” x 3”

5.5. Falcon BLUE MAX Jr. 15 ml Polypropylene Conical Tube

5.6. Falcon BLUE MAX Jr. 50 ml Polypropylene Conical Tube

5.7. Pipet-Lite LTS Pipette L-200XLS+ (or equivalent), adjustable 20-200 µL

5.8. Pipet-Lite LTS Pipette L-1000XLS+ (or equivalent), adjustable 100-1000 µL

5.9. Pipet-Lite LTS Pipette L-20XLS+ (or equivalent), adjustable 0.2-20 µL

5.10. Pipet-Lite LTS Pipette L-2XLS+ (or equivalent), adjustable 0.1-2 uL

5.11. Pipet-Lite Multi Pipette L12-300XLS+, adjustable 20-300 µL

5.12. Rainin LTS 20 tips, 0.1-20 uL

5.13. Rainin LTS 250 tips, 20-250 µL

5.14. Rainin LTS 300 tips, 20-300 uL

5.15. Rainin LTS 1000, 100-1000 µL

5.16. Eppendorf Centrifuge Tubes, 1.5 ml

5.17. Corning 50 ml Reagent Reservoir, Polystyrene

5.18. Thermomixer C, Eppendorf

5.19. Adhesive Plate Sealers

6. REAGENTS

6.1. Formic Acid, Pierce, LC-MS grade Catalog Number 28905

6.1.1. Store at room temperature in Acid Storage Cabinet

6.2. Acetonitrile, OmniSolv Cat., Number AX0142-6

6.2.1. Store at room temperature in Flammable Cabinet

6.3. Methanol, OmniSolv Cat., Number MX0488-1

6.3.1. Store at room temperature in Flammable Cabinet

6.4. Acetone, Richard-Allan Scientific, Reorder Number 9011

6.4.1. Store at room temperature in Flammable Cabinet

6.4.2. Pierce™ Protein G Magnetic Beads (Cat. No. 88847)

6.4.2.1. Store in the refrigerator (4°C)

6.4.3. Pierce™ Protein A Magnetic Beads(Cat. No. 88845)

6.4.3.1. Store in the refrigerator (4°C)

6.5. 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate, CHAPS, Sigma-Aldrich (Cat. Number C9426)

6.6. PBS 10x, pH 7.4, Gibco/Invitrogen, Cat. Number 7001169

7. DEFINITIONS

ESI: Electrospray ionization

MRM: multi- reaction monitoring

8. PROCEDURE

8.1. Preparation of Dilution Buffer

8.1.1. Prepare 1X PBS/CHAPS solution from stock 20X Stock

8.1.1.1. Prepare a 20X stock of PBS/CHAPS (1X PBS/1% CHAPS) as needed

8.1.1.1.1. Weigh out 0.1 g CHAPS and add to 15 mL conical tube

8.1.1.1.2. Add 9 mL DI H2O and 1 mL 10X PBS

8.1.1.1.3. Mix into CHAPS is dissolved

8.1.1.2. Label a 50 mL conical tube “1X PBS/CHAPS”

8.1.1.3. Add 42.5 mL DI H2O

8.1.1.4. Add 2.5 mL 20X stock

8.1.1.5. Add 5 mL 10X PBS

8.1.1.6. Mix well to combine solutions

8.2. **Preparation of Purified Antibodies**

8.2.1. Dilute final antibodies in 1X PBS/CHAPS from stocks to a final concentration of 50 µg/mL.

8.2.2. Prepare sufficient diluted antibody for use in 3 replicates including 10% excess volume in the preparation.

8.2.3. Transfer 100 µL of diluted antibodies into a 96 well plate for a total of 5 µg antibody per replicate.

8.3. Preparation of Antigen Solutions

8.3.1. Dilute light/unlabeled peptide antigens to a final concentration of 700 nM with 1X PBS/CHAPS.

8.3.2. Dilute heavy labeled peptide antigens to a final concentration of 100 nM with 1X PBS/0.05% CHAPS.

8.3.3. Prepare enough of each dilution for 3 replicates per antibody including excess.

8.3.4. Transfer 100 µL of light peptide antigen solution and 25 µL of heavy peptide antigen solution into each well. Final peptide amount added per well is 2 pmol light peptide and 0.5 pmol heavy peptide.

8.4. Preparation of Magnetic Beads

8.4.1. Remove magnetic beads from refrigerator to warm to room temperature and briefly vortex the beads in the vials at the minimum speed to suspend them thoroughly. Protein A beads are used for rabbit antibodies, while Protein G are preferred for mouse antibodies.

8.4.2. Aliquot undiluted beads into a 1.5 mL microcentrifuge tube. Use a sufficient volume to add 10 uL of beads per well (include a 10% excess).

8.4.3. Wash prepared beads before use in the assay.

8.4.3.1. Separate beads from initial dilution using magnetic microtube rack and remove the storage buffer from the beads.

8.4.3.2. Add fresh buffer equivalent to the initial volume of the beads suspension and re-suspend the beads gently with a pipettor to

wash them. Remove the wash buffer as above and repeat this step.

8.4.3.3. Remove the wash buffer from previous step and re-suspend the beads in buffer at the desired final volume (initial volume).

8.4.4. Add 10 µL of washed beads dilution to each well.

8.5. Overnight Incubation of Antibody/Antigen/Magnetic Bead Complex

8.5.1. Add all components of IP mix to a 96 well V-bottom KF plate according to the plate layout. This will be the IP plate.

8.5.2. Seal plate with a plastic or foil adhesive plate sealer ensuring that it is tightly covering the wells so as to keep evaporation to a minimum.

8.5.3. Incubate plate for 12-24 hours in ThermoMixer C with plates adapter at 4°C with shaking to ensure consistent suspension of all components, especially the beads.

8.6. Prepare KingFisher™ Flex for automated run

8.6.1. Load 200 µL of 1X PBS/0.03% CHAPS into each well of a 96 KF V-Bottom Microplate. This is wash plate #1.

8.6.2. Repeat the same 6.2.10.1 for 2 other plates, which will be wash plates #2 and #3.

8.6.3. To guarantee the absence of air bubbles in the plates, spin down all the plates in the Eppendorf centrifuge at 2000 rpm speed, for 2 min at 4°C before proceeding to placing the plates in their proper position on the KingFisher™ Flex.

8.6.4. Stack a Tip Comb for KingFisher™ Flex 96 KF Magnet on a 96 KF V-Bottom Microplate. This is the Tip Comb Plate.

8.6.5. Load 50 µL of 5% Acetic Acid, 3% Acetonitrile solution into each well of a KingFisher 96 KF microplate. Prepare only when necessary to avoid evaporation. This is the Elution Plate.

8.6.5.1. To guarantee the absence of air bubbles in the elution plate, spin down all the plate in the Eppendorf centrifuge at 2000 rpm speed, for 2 min at 4°C before proceeding to placing the plates in their proper position on the KingFisher™ Flex.

8.7. Operate KingFisher™ Flex

8.7.1. Launch BindIt™ Software and select the proper method. The protocol has 9 steps:

8.7.1.1. *Step #1*: **Pick-up: Tip Comb** from Tip Comb Plate

8.7.1.2. *Step #2*: **Collect beads** from IP Plate (collect count: 3; collect time: 1 sec)

8.7.1.3. *Step #3*: **Mixing step:** briefly mix the beads/antibody/antigen complex to ensure it is fully suspended. Mix time: 5 minutes at slow speed. Beads are then collected at the end of this step.

8.7.1.4. *Step #4*: **Wash #1** from Wash plate #1 (release beads at beginning of step, mixing 1.5 min at slow speed, collect beads at end of step, collect beads at end of step)

8.7.1.5. *Step #5*: **Wash #2** from Wash plate #2 (release beads at beginning of step, mixing 1.5 min at slow speed, collect beads at end of step)

8.7.1.6. *Step #6*: **Wash #3** from Wash plate #3 (release beads at beginning of step, mixing 1.5 min at slow speed, collect beads at end of step)

8.7.1.7. *Step #7*: **Elution** from Elution plate (release beads at beginning of step, mixing 5 min at slow speed)

8.7.1.8. *Step #8*: **Release beds** to IP plate (5 s release time at fast speed)

8.7.1.9. *Step #9*: **Leave: Tip Comb** to Tip Comb Plate. Hit the “Start” icon and assign a name for the run indicating the date in which the assay is performed.

8.7.2. Follow the instructions to load the plates on the instrument in the correct order: Tip Comb, Elution, Wash #3, Wash #2, Wash #1, IP plate.

8.7.3. Allow for the run to be completed. It should take about 30 min.

8.7.4. When the run is completed, follow the instructions to unload the plates from the KingFisher™ Flex and cover the Elution plate with a plate sealer immediately.

8.8. Sample preparation for Shimadzu 8050

8.8.1. Transfer all 25 uL of eluate to each plate well using a multichannel pipettor in a v-bottomed 96-well storage plate PCR plate).

8.8.2. Spin down the PCR plate in the Eppendorf centrifuge at 2000 rpm speed, for 2 min at 4°C. Properly balance the centrifuge.

8.8.3. Prepare a randomized sequence of all replicates in excel to be used with the Shimadzu software. Save batches and data to appropriate network drive.

8.8.4. Inject 20 uL for each replicate.

8.8.5. All data will be analyzed with Skyline.

9. REFERENCED DOCUMENTS

9.1. KingFisher Flex - User Manual, Thermo Fisher Scientific, Inc.