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: **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.   Eppendorf Series 2000 Reference Pipettor, adjustable 500-5000 µL

5.8.   Eppendorf Series 2000 Reference Pipettor, adjustable 500-2500 µL

5.9.   Eppendorf Series 2000 Reference Pipettor, adjustable 50-200 µL

5.10.   Eppendorf Series 2000 Reference Pipettor, adjustable 100-1000 µL

5.11.   Eppendorf Series 2000 Reference Pipettor, adjustable 0.2-10 µL

5.12.   Eppendorf Series 2100 Research Multi 8-channel Pipettor, adjustable 30-300 µL

5.13.   Eppendorf Series 2100 Research Multi 8-channel Pipettor, adjustable 0.5-10 µL

5.14.   Eppendorf ep T.I.P.S Reloads Pipette Tips. 500-5000 µL

5.15.   Eppendorf ep T.I.P.S Reloads Pipette Tips. 500-2500 µL

5.16.   Eppendorf ep T.I.P.S Reloads Pipette Tips. 2-200 µL

5.17.   Eppendorf ep T.I.P.S Reloads Pipette Tips, 50-1000 µL

5.18.   Eppendorf ep T.I.P.S Reloads Pipette Tips. 0.2-10 µL

5.19.   Eppendorf Centrifuge Tubes, 1.5 ml

5.20.   Corning 50 ml Reagent Reservoir, Polystyrene

5.21.   Thermomixer C, Eppendorf

5.22.   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)

    6.5.   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 samples for KingFisher™ Flex Magnetic Particle Processor with 96 KF Head.

        8.1.1.   Prepare a worksheet indicating sample placement on particle processor and the PCR plate to be loaded in the autosampler.

    8.2.   Prepare fresh 1X PBS/0.03% CHAPS.

        8.2.1.   Measure 100 mL of PBS 10x in a 1000 mL glass cylinder.

        8.2.2.   Add 18 mega-ohm deionized water to a final volume of 1000 mL.

        8.2.3.   Transfer into a bottle and mix.

        8.2.4.   Dissolve 0.03% CHAPS w/v thoroughly in freshly prepared 1X PBS.

8.3.   Preparation of purified antibodies:

8.3.1.   Dilute final antibodies from stocks to a final concentration of 20 μg/mL. Prepare sufficient diluted antibody for use in 3 replicates including 10% excess volume in the preparation. Dilutions of purified antibodies are done in 1X PBS/0.03% CHAPS.

8.3.2.   Transfer 100 μL of diluted antibodies into a 96 well plate according to the plate distribution as described at step 8.1.1 for a total of 2 μg antibody per replicate.

8.4.   Preparation of Antigen solutions

8.4.1.   Dilute light/unlabeled peptide antigens to a final concentration of 20 nM with 1X PBS/0.03% CHAPS. Prepare sufficient peptide dilution for 3 replicates per antibody and include excess volume in the preparation.

8.4.2.   Dilute heavy labeled peptide antigens to a final concentration of 5 nM with 1X PBS/0.03% CHAPS. As before, prepare enough dilution for 3 replicates per antibody including excess.

8.4.3.   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.

8.5.   Preparation of the Pierce™ Protein G Magnetic Beads

8.5.1.   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.

8.5.2.   Prepare a 1:1 dilution of suspended beads in 1X PBS/0.03% CHAPS in sufficient quantity for all wells. Consider 8 μL of beads diluted suspension/capture.

8.5.3.   Wash prepared beads before use in the assay.

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

8.5.3.2.   Add fresh buffer equivalent to the final desired volume of the beads dilution and re-suspend the beads gently with a pipettor to wash them. Remove the wash buffer as above and repeat this step.

8.5.3.3.   Remove the wash buffer from previous step and re-suspend the beads in buffer at the desired final volume.

8.5.4.   Add 8 μL of washed beads dilution to each well.

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

8.6.1.   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.

8.6.2.   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.

8.6.3.   Incubate plate for 12-24 hours in ThermoMixer C with plates adapter at 4°C with shaking at 1000 rpm to ensure consistent suspension of all components, especially the beads.

8.7.   Prepare KingFisher™ Flex for automated run

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

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

8.7.3.   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.

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

8.7.5.   Load 25 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.

8.7.5.1.   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.

8.8.   Operate KingFisher™ Flex

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

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

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

8.8.1.3.   *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.

8.8.1.4.   *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)

8.8.1.5. *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)

8.8.1.6. *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)

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

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

8.8.1.9. *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.

8.8.2. 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.

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

8.8.4. 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.

8.9. Sample preparation for Shimadzu 8050

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

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

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

8.9.4. Inject 20 uL for each replicate.

8.9.5. All data will be analyzed with Skyline.

9. <u>REFERENCED DOCUMENTS</u>

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