STANDARD OPERATION PROCEDURE FOR THE COMMON FUND: PROTEIN CAPTURE REAGENTS PROGRAM (IP-MS)

1. PURPOSE

This procedure will describe how to prepare human Fab / antigen samples or Mouse Monoclonal Antibody / Antigen samples subjected to robotic characterization and purification with subsequent analysis by MALDI-TOF mass spectrometry.

2. SCOPE

This procedure applies to all samples that are processed for analysis using the KingFisher™ Flex Magnetic Particle Processor and Applied Biosystems Voyager DE-Pro MALDI TOF mass spectrometer, equipped with equipped with a CovalX HM1 detector. This procedure will describe sample preparation and instrument operation for the KingFisher™ Flex Magnetic Particle Processor and Applied Biosystems Voyager DE-Pro MALDI TOF mass spectrometer, equipped with equipped with a CovalX HM1 detector.

3. RESPONSIBILITIES

It is the responsibility of the analyst to follow the procedural steps as written and to document any deviations, problems and observations during an experiment on their notebook.

4. REQUIREMENTS

5. EQUIPMENT

5.1. ABI Voyager – DE Pro MALDI-TOF Mass Spectrometer (or equivalent) equipped with a CovalX HM1 detector

5.2. ABI Voyager MALDI sample plate, Cat. Number V700664

5.3. Mini Vortexer, VWR

5.4. Spectrafuge Mini Centrifuge, Labnet International Inc.

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

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

5.7. KingFisher™ Flex Magnetic Particle Processor with 96 Deep-well Head (Thermo Scientific)

5.8. Tip Comb for KingFisher™ Flex 96-Well Deep Well Magnets (Cat. Number 97002534)

5.9. KingFisher™ Flex 96-Well Deep V-Bottom Microplate, Nonsterile, 2mL (Cat. Number 95040450)

5.10. Hard-Shell® Low-Profile Thin-Wall 96-Well Skirted PCR Plates (Cat. Number HSP-9601)

5.11. Eppendorf Centrifuge 5810R with plate rotor

5.12. Screw Cap with O-ring for Micro Centrifuge Tubes, Bio Plas Inc., Cat. Number 4215

5.13. Mettler AJ100 Analytical Balance

5.14. Weighing Paper, 3” x 3”

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

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

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

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

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

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

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

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

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

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

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

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

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

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

5.29. Eppendorf Centrifuge Tubes, 1.5 ml

5.30. Corning 50 ml Reagent Reservoir, Polystyrene

5.31. DPC MicroMix® 5 Shaker

5.32. Adhesive Plate Sealers.

6. REAGENTS

6.1. Human IgG, Fab Protein, Novus Biologicals (Cat. Number NBP1-97020) for human Fab experiment.

6.2. ChromePure Mouse IgG, whole molecule, Jackson Immuno Research Laboratories (Code 015-000-003) for Mouse IgG experiment.

6.3. Sinapic Acid, Fluka (puriss. P.a. matrix substance for MALDI-MS), Cat. Number 85429

6.3.1. Store at room temperature in Dessicator at 20-30% RH

6.4. Trifluroacetic Acid, Applied Biosystem, Part. No 400003

6.4.1. Store at room temperature in Acid Storage Cabinet

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

6.5.1. Store at room temperature in Flammable Cabinet

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

6.6.1. Store at room temperature in Flammable Cabinet

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

6.7.1. Store at room temperature in Flammable Cabinet

6.8. PureProteome™ Kappa Ig Binder Magnetic Beads, Millipore (Cat. Number LSKMAGKP02) for human Fab experiment.

6.8.1. Store in the refrigerator (4°C)

6.9. PureProteome™ Lambda Ig Binder Magnetic Beads, Millipore (Cat. Number LSKMAGLM02) for human Fab experiment

6.9.1. Store in the refrigerator (4°C)

6.10. Dynabeads® Protein G (Cat. Number 10004D) for the Mouse IgG experiment.

6.10.1. Store in the refrigerator (4°C)

6.11. Octyl–β–Glucoside (BOG), Pierce, Cat. Number 28309

6.11.1. Store in the refrigerator (4°C)

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

7. DEFINITIONS

MALDI-TOF: matrix assisted laser desorption ionization time-of-flight

8. PROCEDURE

8.1. Preparation of samples for KingFisher™ Flex Magnetic Particle Processor with 96 Deep-well Head.

8.1.1. Prepare a worksheet indicating sample placement on particle processor and the MALDI plate.

8.1.2. Include one control for each target (Human IgG, Fab Protein for the human Fab; ChromePure Mouse IgG, whole molecule for the Mouse IgG experiment).

8.1.3. Set aside n+1 spots (where n = # of antigens) for confirmation of starting antigen material and calibration compound mixture (BSA/myoglobin).

8.2. Prepare fresh 2X PBS.

8.2.1. Measure 100 mL of PBS 10x in a 500 mL glass cylinder.

8.2.2. Add 18 mega-ohm deionized water to a final volume of 500 mL.

8.2.3. Transfer into a bottle and mix.

8.3. Prepare fresh 1mM BOG (It is possible to use a previously prepared 1mM BOG solution as long as the solution is not older than one week)

8.3.1. Weigh 146.2 mg of BOG, and dissolve in 500 mL of 18 mega-ohm deionized water, mix well.

8.4. Prepare 1X PBS /500 μM BOG.

8.4.1. Mix 250 mL of 2X PBS solution and 250mL of 1mM BOG. This buffer that will be used in the wash steps, bead dilution and the dilution of antibody and antigen solutions.

8.5. Preparation of human Fab solutions

8.5.1. Dilute human Fab solutions or Mouse IgG, including relative controls, to a concentration of 50 μg/mL with 1X PBS/500 μM BOG.

8.5.2. Transfer 200 μL of human Fab or Mouse IgG solutions into a labeled 1.5 mL conical microcentrifuge tube.

8.6. Preparation of Antigen solutions

8.6.1. Dilute antigen solutions to a concentration of 100 μg/mL with 1X PBS/500 μM BOG.

8.6.2. Transfer 300 μL of antigens solutions into a labeled 1.5 mL conical microcentrifuge tube.

8.7. Pre-incubation human Fab / antigen or Mouse IgG / antigen

8.7.1. For the human Fab experiment, mix 100 μL of Antigen solution and 100 μL of the correspondent human Fab or the Fab protein from Novus (negative control) in a 96-Well Deep V-Bottom Microplate. For the mouse IgG experiment, mix 100 μL of Antigen solution and 100 μL of the correspondent Mouse IgG or the Mouse IgG whole molecule from Jackson Immuno Research Laboratories (negative control) in a 96-Well Deep V-Bottom Microplate.

8.7.2. Cover the mixture and incubate on the DPC MicroMix® 5 Shaker (set at 4) for 2 hours at room temperature to facilitate the formation of the Fab-antigen complex or the Mouse IgG-antigen. This is the IP-Mix Plate.

8.8. Preparation of the PureProteome™ Kappa and Lambda Ig Binder Magnetic Beads

8.8.1. Remove magnetic beads from refrigerator to warm to room temperature and briefly vortex the beads in the vials at the minimum speed.

8.8.2. For the human Fab experiment, dilute each bead solution 1 in 40 in 1X PBS/500 μM BOG. Final volume is 100 μL/well plus extra 10% volume. For example, for a full 96 well plate Final volume will be 96*100 μL *1.1 = 10560 μL. Therefore mix 10,032 μL with 264 μL of Kappa Ig binder beads and 264 μL of Lambda Ig binder beads. For the Mouse IgG experiment, dilute the Protein G beads 1 in 4 in 1X PBS/500 μM BOG. Final volume is 100 μL/well plus extra 10% volume. For example, for a full 96 well plate Final volume will be 96*100 μL *1.1 = 10560 μL. Therefore mix 8448 μL with 2112 μL of Protein G beads.

8.8.3. With a Multi 8-channel Pipet aliquot 100 μL of the magnetic bead suspension into each well of in a 96-Well Deep V-Bottom Microplate. This is the K and L beads plate or the Protein G plate.

8.9. Prepare KingFisher™ Flex for automated run

8.9.1. Load 200 μL of 1X PBS/500 μM BOG into each well of a 96-Well Deep V-Bottom Microplate. This is the Pre-wash plate #1.

8.9.2. Repeat the same 6.2.10.1 for other 3 plates that will be respectively Pre-wash plate #2, Wash plate #1 and Wash plate #1

8.9.3. Stack a Tip Comb for KingFisher™ Flex 96-Well Deep Well Magnets on a 96-Well Deep V-Bottom Microplate. This is the Tip Comb Plate.

8.9.4. Load 25 μL of 5% Acetic Acid, 3% Acetonitrile solution into each well of a 96-Well Deep V-Bottom Microplate. This is the Elution Plate.

8.9.5. To guarantee the absence of air bubbles in the DW 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.

8.10. Operate KingFisher™ Flex

8.10.1. Launch BindIt™ Software and select “Human fab Protocol” or “Mouse IgG Protocol”. The protocol has 10 steps:

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

8.10.1.2. *Step #2*: **Collect beads** from K and L beads plate or the Protein G plate (collect count: 3; collect time: 1 sec)

8.10.1.3. *Step #3*: **Pre-wash #1** from Pre-wash plate #1 (release beads at beginning of step, mixing 1 min at medium speed, collect beads at end of step, collect beads at end of step)

8.10.1.4. *Step #4*: **Pre-wash #2** from Pre-wash plate #2 (release beads at beginning of step, mixing 1 min at medium speed, collect beads at end of step)

8.10.1.5. *Step #5*: **Fab-Target Complex IP** or from **Mouse IgG-Target Complex IP** IP-Mix Plate (release beads at beginning of step, mixing 30 min at medium speed, collect beads at end of step)

8.10.1.6. *Step #6*: **Wash #1** from Wash plate #1 (release beads at beginning of step, mixing 1 min at medium speed, collect beads at end of step)

8.10.1.7. *Step #7*: **Wash #2** from Wash plate #2 (release beads at beginning of step, mixing 1 min at medium speed)

8.10.1.8. *Step #8*: **Elution** from Elution plate (release beads at beginning of step, mixing 10 min at medium speed)

8.10.1.9. *Step #9*: **Release beds** to K and L beads plate (5 s release time at fast speed)

8.10.1.10. *Step #10*: **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 (this step can be omitted).

8.10.2. Follow the instrument instructions to load the plates on the instrument in the correct order: Tip Comb, Elution, Wash #2, Wash #1, IP mix, Pre-wash #2, Pre-wash #1, K and L beads.

8.10.3. Allow for the run to be completed. It should take about 50 min.

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

8.11. Wash and prepare ABI MALDI plate.

8.11.1. Rinse the plate thoroughly with 70% ethanol. Wipe the plate dry using a Kimwipe. Repeat.

8.11.2. As a second wash, rinse the plate thoroughly with methanol. Wipe the plate dry with a Kimwipe. Repeat.

8.11.3. As a third wash, rinse the plate thoroughly with Acetone. Wipe the plate dry with a Kimwipe. Repeat.

8.11.4. Cover plate to protect from dust until use.

8.12. Prepare Matrix Solution.

8.12.1. Prepare a saturated solution of Sinapic Acid (SA) and add 20 mL of 0.1% TFA/acetonitrile 50/50 (v/v) in Screw Cap with O-ring for Micro Centrifuge Tubes. This is the Matrix Solution and the solution should be saturated with visible precipitate.

8.12.2. Prepare the Matrix Solution just prior to use for the manual spotting procedure. Solution shelf life maximum is 2 hours or keep refrigerated at 4°C for maximum a week.

8.13. Prepare samples for spotting on ABI MALDI plate

8.13.1. Spin down all the Elution Plate in the Eppendorf centrifuge at 2000 rpm speed, for 2 min at 4°C. Properly balance the centrifuge.

8.13.2. With a multi-channel pipette transfer the 25 μL of the eluates to a 96-Well skirted PCR plate.

8.13.3. Spin down all the Elution Plate in the Eppendorf centrifuge at 2000 rpm speed, for 2 min at 4°C. Properly balance the centrifuge.

8.14. Spot samples

8.14.1. Spot the two positions on the plate with a standard mixture of Myoglobin and BSA (10 μM Myoglobin, 100 μM BSA, 1:1 v/v in 0.1%TFA), mixing 0.75-1 μL of standard mixture and same volume of SA matrix.

8.14.2. Spot each sample mixing 0.75-1 μL of sample and same volume of SA matrix as indicated in the worksheet as described in section 8.1.1.

8.14.3. Allow the plate to dry undisturbed at room temperature.

8.15. ABI Voyager – DE Pro Mass Spectrometer Procedure

8.15.1. Check the status of the system and check ion source chamber and flight tube (mirror chamber) pressures via Voyager Control software. The ion source pressure should be below 1 x 10^{-6} torr and mirror chamber below 9 x 10^{-7} torr before starting analysis.

8.15.2. Launch the Voyager Control software and load previously prepared MALDI plate.

8.15.3. Check the status Covalx box:

8.15.3.1. Switch the Covalx box on;

8.15.3.2. Verify high mass option is selected;

8.15.3.3. Verify voltages are properly set: HV1 at 2.50, HV2 at 20.00.

8.16. Check the status of the default calibration:

8.16.1. Launch the default calibration application from the desktop shortcut.

8.16.2. Select linear mode.

8.16.3. Verify value for LFF Len (mm) is 1418.4.

8.17. Perform calibration:

8.17.1. Acquire and save a spectrum from the calibration spot using the acquisition method for analysis is “HMD – BSA – automated”. (path: V:\Simona\methods\ HMD – BSA – automated)

8.17.2. Launch Voyager Data Explorer software

8.17.3. Select the calibration spectrum, myoglobin/BSA.

8.17.4. The spectrum should then be manually calibrated by selecting the BSA (66431 amu) and myoglogin (16952 amu) standards from the reference list within the processing software.

8.17.5. The calibration file is then saved and is to be used for processing of subsequent spectra.

8.18. Compile sequence for automated acquisition:

8.18.1. Launch Sequence software via icon in Voyager Control software. Note: a plate must be loaded into the instrument to edit Sequence information.

8.18.2. The acquisition method for analysis is “HMD – BSA – automated”. (path: V:\Simona\methods\ HMD – BSA – automated)

8.18.3. Fill sequence sample information from sample worksheet used for the automated preparation of samples as indicated in the worksheet described in section 8.1.1. Care should be taken to maintain sample order and description.

8.18.4. Select for each sample to be run the calibration option as “external” and the calibration file saved as described in section 8.17.5.

8.18.5. Before starting the sequence, under the Sequence Control panel, go to “View”, then to “Preferences”. In the “Preferences” section make sure the “enable low memory” dialog box is *unchecked*. A known bug in the software will not allow the sequence to proceed if this box is checked.

8.18.6. Confirm that “HMD – BSA – automated” is the loaded method in the Voyager Control Software, and start the sequence by selecting “start sequence” under the “Control” section, or by pressing the “play (triangle)” icon.

8.18.7. A full plate of 100 spots will take approximately 2-3 hours to run, so every effort should be made to have the analysis run overnight.

8.19. Data analysis is performed with Voyager Data Explorer software.

8.19.1. Spectra are already calibrated.

8.19.2. The resulting spectra should be saved as PowerPoint / PDF files for future use, indicating the file name and acquisition date.

8.19.3. All MALDI raw data and spectral analysis data should be saved on a server with regular backup procedures in operation.

9. REFERENCED DOCUMENTS

9.1. 7.1 KingFisher Flex - User Manual, Thermo Fisher Scientific, Inc.