STANDARD OPERATING PROCEDURE

Title: Robotic Assay Procedure for Antibody/Antigen Characterization with MALDI-TOF Mass Spectrometric Detection

SOP#: M-120

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1. PURPOSE

The purpose of this document is to describe how to prepare 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 Hamilton Star Liquid Handling Robot and Applied Biosystems Voyager DE-Pro MALDI TOF mass spectrometer. This procedure will describe sample preparation and instrument operation for the Hamilton Star robot and Voyager – DE Pro mass spectrometer.

3. RESPONSIBILITIES

It is the responsibility of person(s) performing this procedure to be familiar with lab safety procedures. The interpretation of results must be done by a person trained in the procedure and familiar with such interpretation. 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 in their notebook.

4. EQUIPMENT

- ABI Voyager – DE Pro MALDI-TOF Mass Spectrometer (or equivalent)
- ABI Voyager MALDI sample plate (Cat. # V700664)
- Sample table for ABI MALDI plate (modified), LC Packings (Cat. #1000-61)
- Mini Vortexer, Model VM-3000 (VWR, Cat. #58816-121)
- Spectrafuge Mini Centrifuge (Labnet International Inc., Cat. #C1301B)

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- Dynal MPC 96S (Invitrogen, Cat. #120.27)
- Hamilton Microlab STAR Liquid Handling Robot (or equivalent)
- Mettler AJ100 Analytical Balance
- Eppendorf Series 2000 Reference Pipettor, adjustable 500-5000 µL
- Eppendorf Series 2000 Reference Pipettor, adjustable 500-2500 µL
- Eppendorf Series 2000 Reference Pipettor, adjustable 50-200 µL
- Eppendorf Series 2000 Reference Pipettor, adjustable 100-1000 µL
- Eppendorf Series 2000 Reference Pipettor, adjustable 0.2-10 µL
- Eppendorf Series 2100 Research Multi 8-channel Pipettor, adjustable 30-300 µL
- Eppendorf Series 2100 Research Multi 8-channel Pipettor, adjustable 0.5-10 µL
- MicroMix® 5 Shaker (EURO/DPC Ltd., Cat. #MMIX5)

5. MATERIALS

- Standard 300 µl CO-RE tips for the Hamilton Microlab STAR (Hamilton, Cat. #235902)
- Low Volume Tips (10 µl) without Filters for the Hamilton Microlab STAR, (Hamilton, Cat. #235900)
- 12 Column Reservoir Pyramid Bottom (Innovative Microplate, Cat. #S30016)
- 96 Well, Reservoir Low Profile Pyramid Bottom (Innovative Microplate, Cat. #S30018)
- Universal Lid without corner notch (Corning Incorporated, Costar, Cat. #3098)
- Storage Plate, V-bottom 96 Well (Abgene House, Cat. #AB-1058)
- Storage Plate, V-bottom 96 Well (Corning Incorporated, Costar, Cat. #3357)
- Micro Centrifuge Tubes, 0.5 ml conical, upright (Bio Plas, Inc., Cat. #4200)
- Screw Cap with O-ring for Micro Centrifuge Tubes (Bio Plas Inc., Cat. #4215)
- Weighing Paper, 3” x 3”
- Falcon BLUE MAX Jr. 15 ml Polypropylene Conical Tube (Cat. #352097)
- Falcon BLUE MAX Jr. 50 ml Polypropylene Conical Tube (Cat. #352098)

- Eppendorf ep T.I.P.S Reloads Pipette Tips. 500-5000 µL (Cat. #022492080)
- Eppendorf ep T.I.P.S Reloads Pipette Tips. 500-2500 µL (Cat. #022491571)
- Eppendorf ep T.I.P.S Reloads Pipette Tips, 50-1000 µL (Cat. #022491555)
- Eppendorf ep T.I.P.S Reloads Pipette Tips, 20-300 µL (Cat. #022491539)
- Eppendorf ep T.I.P.S Reloads Pipette Tips. 2-200 µL (Cat. #022491539)
- Eppendorf ep T.I.P.S Reloads Pipette Tips. 0.1-10 µL (Cat. #022491547)
- Corning 50 ml Reagent Reservoir, Polystyrene (Cat. #4870)
- Plastic Vials, 7 mm (Biacore, Cat. #BR-1002-12)
- Polystyrene 10 mL pipettes (FALCON, Cat. #356551)
- Recombinant Human Thyroid Stimulating Hormone (TSH) (Seradyn, Inc, Cat. #ABT0351)
- Monoclonal Anti-Thyroid Stimulating Hormone (TSH) (Seradyn, Inc., Cat. #MIT0412B)
- Beta-2-microglobulin from human urine (Sigma-Aldrich, Cat. #M4890)
- Purified anti-Human beta-2-Microglobulin, Biolegend, Cat. #316302)
- SuperBlock Blocking Buffer in PBS (Pierce, Cat. #37515)
- Sinapic Acid, (puriss. P.a. matrix substance for MALDI-MS), (Fluka, Cat. #85429)
    - Store at room temperature in Dessicator at 20-30% Relative Humidity (RH)
- Trifluroacetic Acid (Applied Biosystem, Cat. #400003)
    - Store at room temperature in Acid Storage Cabinet
- Acetonitrile (OmniSolv, Cat. #AX0142-6)
    - Store at room temperature in Flammable Cabinet
- Methanol (OmniSolv, Cat. #MX0488-1)
    - Store at room temperature in Flammable Cabinet
- Acetone (Richard-Allan Scientific, Cat. #9011)
    - Store at room temperature in Flammable Cabinet
- Dynabeads® Pan Mouse IgG (Dynal, Invitrogen Bead Separation, Cat. Number 110-41/Cat. #110.42)

- Octyl–β–Glucoside (BOG), (Pierce, Cat. #28309)
  - Store in the refrigerator (4°C)
- PBS 10x, pH 7.4 (Gibco/Invitrogen, Cat. #7001169)
- Mineral oil (Fluka, Cat. #69794)
- Chloroform (Fisher Scientfic, Cat. #C298-500)

6. REAGENTS

- Various antibodies from NCI Clinical Proteomic Technologies for Cancer program to be tested.

7. PROCEDURE

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

7.2. Preparation of samples for Hamilton Robot and MALDI

7.2.1. Prepare a worksheet indicating sample placement on robot and the MALDI plate.

- Include one blank (no mAb) and one B2M-antiB2M as positive control sample in each run. All samples, blank, positive and negative controls are run in duplicate.
- Set aside n+1 spots (where n = # of antigens) for confirmation of starting antigen material and calibration compound mixture (50 μM BSA/5μM Myoglobulin)

7.2.2. Wash, prepare, mount ABI MALDI plate.

- First, rinse the plate thoroughly with 70% ethanol. Wipe the plate dry using a Kimwipe. Repeat.
- As a second step, rinse the plate thoroughly with 100% methanol. Wipe the plate dry with a Kimwipe. Repeat.
- As a third step, rinse the plate thoroughly with 100% acetone. Wipe the plate dry with a Kimwipe. Repeat.
- Mount MALDI plate on sample table by pressing plate firmly into pre-cut depression using a Kimwipe to prevent contamination of the surface.
- Lightly coat MALDI plate with solution of mineral oil/chloroform (1:1, v/v) using a cotton tipped applicator.
- Cover plate with a plastic lid to protect from dust until use.

7.2.3. Prepare fresh 2X PBS.

- Measure 100 mL of 10X PBS in a 500 mL glass cylinder.
- Add 18 mega-ohm deionized water to a final volume of 500 mL.
- Transfer into a bottle and mix.

7.2.4. Prepare fresh 1mM Octyl–β–Glucoside (BOG) (It is possible to use a previously prepared 1mM BOG solution as long as the solution is not older than one week at 4°C)

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

7.2.5. Prepare 1X PBS /500 μM BOG.

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

7.2.6. Preparation of Antibody solution

- Dilute antibody solution, including anti-TSH and anti-B2M controls, to a concentration of 62.5 ng/μL with 1X PBS/500 μM BOG. Calculate 160 μL for each vial.
- Transfer 150 μL of antibody solution into a labeled 0.5 conical microcentrifuge tube.

7.2.7. Preparation of Antigen solution

- Dilute antigen solution to a concentration of 200 ng/μL with 1X PBS/500 μM BOG. Calculate 160 μL for each vial.
- Positive control antigen, B2M should be diluted to a concentration of 100 ng/μL. Calculate 160 μL for each vial.
- Transfer 150 mL of antigen solution into a labeled 0.5 mL microcentrifuge tube.

7.2.8. Preparation of the Dynabeads Pan Mouse IgG beads.

- Add 3 mL of Dynabeads Pan Mouse IgG beads to 27 mL 1X PBS/500 μM BOG in a Falcon BLUE MAX Jr. 50 mL conical vial, mix well. The final concentration of the beads suspension will be 10^7 beads/mL.
- Using a Multi 8-channel Pipet aliquot 250 μL of the magnetic bead suspension into each well of a 96- well Costar V bottom storage plate and place the plate in the position “Shaker” on the robot deck. This operation should be performed immediately to prevent beads settling.

7.2.9. Prepare Matrix Solution.

- Weigh 0.2 g of Sinapic Acid (SA) and add 20 mL of 0.1% Trifluroacetic Acid (TFA)/acetonitrile 70/30 (v/v) in a Falcon BLUE MAX Jr 50mL conical vial, mix well. This is the Matrix Solution. A small amount of precipitate may be visible, this is acceptable.
- Prepare the Matrix Solution just prior to use on the robot. Solution shelf life maximum is 2 hours at room temperature.

7.2.10. Prepare a 1:25 dilution of SuperBlock in water

- Measure 192 mL of water and transfer in a clean glass bottle.
- Add 8 mL of SuperBlock to the water, mix thoroughly

7.2.11. Prepare the Hamilton liquid handling robot to distribute the antibody samples and to run the first part of the method: binding of the antibodies to the Pan Mouse IgG beads.

Distribution of Antibody Samples

- Place 5 racks of Standard Volume (300 µl) tips (96) on Carrier 1 of the Hamilton.
- Place 4 racks of Standard Volume (300 µl) tips (96) starting at the position furthest back and moving forward on Carrier 5.
- Place 2 racks of Standard Volume (300 µl) tips (96) starting in the front position on Carrier 4 and place 2 racks of Standard Volume (300mL) tips starting at the position furthest back moving forward on the same carrier.
- Place the Antibody vials (7.2.6) in the Sample Carriers in the order corresponding to the worksheet (7.2.1). If there are not 48 samples, use water blanks to fill the empty spaces on the Sample Carriers.
- Pour at least 200 mL of PBS 1x/500 µM BOG into a 12 columns pyramid bottom reservoir in the position “Buffer” on the robot deck (5th from the front position).
- Place a V-bottom 96-well storage plate in the position “Ab/Ag” on the robot deck (4th from the front position).
- Place an empty 96-well Costar V bottom storage plate with a lid in the position “empty plate” (2nd from the front position).
- Pour at least 100 mL of SuperBlock 1:25 dilution (7.2.10) into a 12 columns pyramid bottom reservoir “SuperBlock/Water” (1st from the front position).
- Place the 96-well magnet in the position “Magnet” on the robot deck.

- Place the 96-well plate containing the previously prepared Pan Mouse IgG bead suspensions in the position “Shaker” on the robot deck.
- Select the “**AbAg_Part_1**” program in the “MoAbs Initiative/IP-MS” folder under the Microlab STAR Method Editor program.
- Click on the Run Control icon (looks like traffic light) to start the program analysis.
- Click on the Green arrow icon at the top of the Hamilton Run Control window to start the program.
- When the first part of the method is over, prepare the Hamilton robot to distribute the antigen samples and to run the second part of the method.

Distribution of Antigen Samples

- Place 5 racks of Standard Volume (300 µl) tips on the Hamilton on Carrier 1.
- Place 3 racks of Standard Volume (300 µl) tips starting at the position furthest back and moving forward on Carrier 2. On the same Carrier place 1 rack of Standard Volume (300 µl) tips in the front position.
- Place 1 rack of Standard Volume (300 µl) tips in the front position on Carrier 4.
- Place 4 racks of Small Volume (10 µl) tips starting in the front position on Carrier 5.
- Place the Antigens vials (7.2.7) in the Sample Carriers in the same order as they are listed on the worksheet. If there are not 48 samples, use water blanks to fill the empty spaces on the Sample Carriers.
- Pour at least 200 mL of PBS 1x/500 µM BOG into a 12 columns pyramid bottom reservoir in the position “Buffer” on the robot deck (5th from the front position).
- Pour at least 200 mL of deionized water into a new 12 columns pyramid bottom reservoir and place on the position “SuperBlock/Water” on the robot deck.
- Place a new V-bottom 96-well storage plate in the position “Ab/Ag” on the robot deck (4th from the front position).
- Place the ABI MALDI plate with holder in the position “MALDI target” on the robot deck (3rd from the front position).

- Place a V-bottom 96-well storage plate in the position “Elution” (#AB-1058) on the robot deck (2nd from the front position).
- Pour Matrix Solution into a low profile pyramid buffer reservoir (waffle) in the position “SA Matrix” on the robot deck. Put lid on.
- Leave the 96-well magnet in the position “Magnet” on the robot deck from the previous method.
- Leave the beads plate in the “Shaker” position from the previous method.
- Select the “**AbAg_Part_2**” program in the “MoAbs Initiative/IP-MS” folder under the Microlab STAR Method Editor program.
- Click on the Run Control icon (looks like traffic light) to start the program analysis.
- Click on the Green arrow icon at the top of the Hamilton Run Control window to start the program.
- After the loading of the antigens onto the beads plate, take the beads plate and transfer it in the cold room (4°C) for 1 hr. incubation on the DPC MicroMix® 5 Shaker (Form:20; Amplitude: 0.5; Time: 60).
- After the incubation, return the plate to the shaker position and hit “Stop Timer” on the computer.
- The Hamilton robot program will provide a spotted MALDI target plate in about 160 min.
- Spot the position 99 and 100 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).
- Allow the plate to dry undisturbed at room temperature for at least 10 minutes or as long as needed for the spots to be completely dry.

7.3. ABI Voyager – DE Pro Mass Spectrometer Procedure

- 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.
- Load MALDI plate with samples spotted from robot along with starting antigen materials and myoglobin/BSA calibrant into instrument. Determine that ion source and mirror chamber pressures achieve above stated minimums.

- Launch Sequence software via icon in Voyager Control software. Note, a plate must be loaded into the instrument to edit Sequence information.
- The acquisition method for analysis is “antigen-auto”. (path: V:\antibody-initiative\methods\antigen-auto)
- Edit Sequence sample information from sample worksheet used for the robotic preparation of samples. Care should be taken to maintain sample order and description.
- Once sample information is entered, print a hardcopy of the sequence information.
- 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.
- Confirm that “antigen-auto” is the loaded method in the Voyager Control Software, and start the sequence by selecting “start sequcence” under the “Control” section, or by pressing the “play (triangle)” icon.
- 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.
- Data analysis is preformed with Voyager Data Explorer software.
- The calibration spectrum, myoglobin/BSA, is first selected.
- The data should be “smoothed” by selecting “Noise Filter/Smooth” under the “Process” heading. Use the “default” method with mass peak resolution at 2000.
- 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.
- The calibration file is then saved and is to be used for processing of subsequent spectra.
- Processing of subsequent spectra are as described above, ie, the data is first smoothed using the default setting, and then calibrated using the calibration file created from the within run analysis. Note, calibration files from previous dates cannot be used for samples analyzed on different dates.

- The resulting spectra should be saved as PDF files for future use.
- All MALDI raw data and spectral analysis data should be saved on a server with regular backup procedures in operation.

8. REFERENCED DOCUMENTS

8.1. Operational Manual, Hamilton Microlab STAR

8.2. Software Program, Hamilton STAR (available upon request from the Antibody Characterization Laboratory, SAIC – Frederick, Building 469, Frederick, MD

8.3. Operation Manual, Applied Biosystems Inc., Voyager DE-Pro MALDI Mass Spectrometer