STANDARD OPERATING PROCEDURE

Title: Evaluation of monoclonal antibody pairing activity using purified cognate antigens by Surface Plasmon Resonance (ProteOn XPR36)

SOP#: M-125

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1. PURPOSE

This procedure will measure the antibody pairing activity of hybridoma supernatants and/or purified monoclonal antibodies with their purified cognate antigens using Surface Plasmon Resonance (SPR) Spectroscopy (BIO-RAD ProteOn XPR 36).

2. SCOPE

This document describes the procedure for all sample analysis using SPR Spectroscopy, ProteOn XPR 36. This procedure will describe sample preparation and evaluation on the ProteOn XPR 36.

3. RESPONSIBILITIES

It is the responsibility of the person(s) performing this analysis to be familiar with laboratory safety procedures and to have basic laboratory skills, specifically trained in the operation and maintenance of the ProteOn XPR36. All procedural steps are to be followed as written and any deviations, problems and observations during an experiment must be documented. The interpretation of the results must be done by a person trained in the procedure and familiar with such interpretation.

4. EQUIPMENT

- ProteOn XPR36, BioRad Protein Interaction Array System
- ProteOn Standard microplate 96 wells BioRad, Cat. #176-6020
- Mettler AJ100 Analytical Balance
- Rainin Pipet plus LTS Pipettor, adjustable 100-1000 µL, Cat. # RL1000
- Rainin Pipet plus LTS Pipettor, adjustable 20-200 µL, Cat. # RL200

- Rainin Pipet plus LTS Pipettor, adjustable 2-20 µL, Cat. # RL20
- Rainin Pipet plus LTS Pipettor, adjustable 0.1-2 µL, Cat. # RL2
- Mini Vortexer, Vortex genie 2 Fisher, Cat. # 12-812.

5. MATERIALS

- ProteOn microplate foils BioRad, Cat. #176-6040
- ProteOn sample vials 1.5ml, with piercable caps, BioRad, Cat. # 176-6010
- Weighing Paper, 3" x 3", VWR, Cat. #28498-002
- Cryogenic freezer boxes, VWR, Cat. #82007-162
- Rainin Space Saver LTS Reloads Pipette Tips. 1000 µL Cat. #GPS-L10
- Rainin Space Saver LTS Reloads Pipette Tips. 250µL Cat. #GPS-L250
- Rainin Space Saver LTS Reloads Pipette Tips. 20 µL Cat. #GPSL-1000
- Eppendorf Centrifuge Tubes, 1.5 ml, VWR, Cat. #21008-953
- Polystyrene 10 mL pipettes, Falcon, Cat. # 356551
- Polystyrene 25 mL pipettes Diagger, Cat. #1BSPS25
- Surfactant –Amps 20 Tween 20 detergent 10% solution Thermo Scientific, Cat. #28320
- Albumin, Bovine fraction V (BSA) Sigma, Cat. # A2153
- ProteOn Amine Coupling Kit BioRad, Cat. # 176-2410
- ProteOn Acetate Buffer pH 4.5 BioRad, Cat. # 176-2121
- ProteOn Maintenance kit BioRad, Cat. # 176-4100
- ProteOn GLM Sensor chip BioRad, Cat. #176-5012
- ProteOn (running buffer) PBS/Tween 2L BioRad Cat. # 176-2720
- ProteOn (running buffer) bottle 2L BioRad Cat. # 176-2700
- 1M Hepes Buffer Solution Invitrogen/Gibco, Cat. # 15630
- Sodium Chloride Solution, BioRad, Cat. #176-2770
- Phosphoric Acid, BioRad, Cat. #176-2260
- Sodium Hydroxide Solution, BioRad Cat. #176-2230
- Hydrochloric Acid, BioRad, Cat. #176-2250

- Sodium Dodecyl Sulfate solution (SDS), BioRad, Cat. #176-2240
- Mouse IgG protein A purified Lampire Biological Laboratories, Cat. #7404304
- Rabbit Anti-Mouse IgG Fc, Jackson ImmunoResearch, Cat. #315-005-046

6. REAGENTS

- Antigen corresponding to the antibody.
- Purified monoclonal antibody corresponding to the Antigen

7. DEFINITION

- GLM Sensor Chip: Medium Binding Amine Coupling Sensor Array
- RAMc: Rabbit Anti-Mouse IgG Fc specific Antibody

8. PROCEDURE

8.1. Preconditioning the Chip

8.1.1. Remove the GLM sensor chip from the refrigerator and warm to room temperature (~30 minutes) and insert chip into ProteOn. The instrument temperature is set to 25°C.

8.1.2. Prepare 1L - 10X stock Hepes Buffered Saline (HBS) stock: (1M Hepes, 1.5 M NaCl)

- 300 mls of 5M NaCl
- 100 mls of 1M Hepes pH 7.55.
- Adjust to 1L with deionized water

8.1.3. Prepare 1L of 1X HBS:

- 100 mls of 10x HBS stock
- Adjust to 1L with deionized water

8.1.4. Prepare the Antibody Characterization Buffer (1X HBS, with 0.005% (v/v) Tween 20 and 0.1% BSA (w/v)):

- 200 mls 10X HBS
- 2 grams BSA
- 1 ml of 10% (v/v) Tween 20 solution
- Adjust to 2L with deionized water
- Add buffer to polypropylene ProteOn bottle

8.1.5. Place ProteOn buffer line A into the 1X HBS. Place ProteOn buffer line B into the Antibody Characterization Buffer.

8.1.6. *Preconditioning Step 1:* Three (3) separate injections are run in the vertical direction in the following order: 0.5% SDS, 50 mM NaOH and 100 mM HCL. All injections are regeneration injections at 30 μl/min and 60 sec injection.

8.1.7. *Preconditioning step 2*: The same reagents described previously in preconditioning step 1 are run in the horizontal direction: 0.5% SDS, 50 mM NaOH and injection followed by 100 mM HCL. All injections are regeneration injections at 30 μl/min and 60 sec injection.

8.1.8. Prepare a 96 deep well plate as follows:

- Add 1 ml of 0.5% SDS to row H wells 1-6
- Add 1 ml of 50 mM NaOH to row G wells 1-6
- Add 1 ml of 100 mM HCl to Row F wells 1-6

8.2 RAMc chip build

8.2.1 Remove ProteOn Amine coupling kit from -20 freezer.

8.2.2 Remove EDAC and sulfo-NHS from ProteOn Amine Coupling Kit. Add 7.5 mls of deionized water to each amber bottle, cap tightly and vortex until in solution.

8.2.3 Label seven 1 ml eppendorf tubes: “EDAC and date” and store in a cryobox at -20°C.

8.2.4 Label seven 1 ml eppendorf tubes: “sulfo-NHS and date” and store in a cryobox at -20°C.

8.2.5 In ice bucket place 1 amber bottle each of reconstituted EDAC and sulfo-NHS (from 8.2.2).

8.2.6 Pre-aliquot 20 µl of RAMc (stock concentration 1.8 mg/ml) in 1 ml eppendorf tubes store in a cryobox in the -20°C freezer.

8.2.7 Remove an eppendorf RAMc from the -20°C freezer and prepare a 10 μg/ml solution of RAMc in 10mM sodium acetate buffer pH 5.0.

- Add 1470 μl of 10 mM Sodium Acetate buffer pH 5.0 and 8.3 μl of RAMc to a 1.5 ml eppendorf tube. Vortex well to mix.

8.2.8 Prepare the 96 deep well plate as follows:

- Add 250 μl RAMc ligand solution in Row D wells 1-6

- Add 250 µl 1M Ethanolamine solution from the amine coupling kit in Row C wells 1-6
- Add 400 µl of 100 mM HCl solution to row F wells 1-6
- Add 125 µl each of EDAC and sulfo-NHS solution to Row E wells 1-6

8.3 Antibody-antigen preparation

8.3.1 Remove antigens and corresponding antibodies to be evaluated from refrigerator.

8.3.2 Supernatants are evaluated undiluted and Purified Antibodies are evaluated at 10 ug/ml.

8.3.3 Determine the molarity of the Antigen stock. For example if the antigen concentration is 2.4 mg/ml you divide the concentration by the molecular weight (i.e. 25700 Da) and the resulting molarity would be 2.4/25700 is 0.0000093385 or 93µM.

8.3.4 Three concentrations of the antigen will be evaluated in each experiment (i.e. 100 nM, 500 nM and 1 µM). From the stock concentration a dilution factor is determined to obtain a working concentration of 1 µM. A minimum volume of 1500 µl is required for the method.

8.3.5 Example dilution chart:

Concentration	dilution factor	previous	buffer	total
1000n	1:93	16.1	1483.9	1500
500n	1:2	1000	1000	2000
100n	1:5	400	1600	2000

8.3.6 Prepare the 96 deep well plate as follows using the plate prepared previously (8.2.8):

- Add 1 ml of Antibody Characterization Buffer and five different monoclonal antibodies (or Supernatants) to Row H wells 7-12
- Add 300 µl Antigen solution to Row G wells 7-12
- Add 300 µl Antigen solution to Row E wells 7-12
- Add 300 µl Antigen solution to Row D wells 7-12

- Add 500 µl mouse IgG Protein A purified solution to Row F wells 7-12

8.3.7 Open ProteOn method and for this system it is in data_Whiteley SOP/open/new method/browse at bottom of screen locate shared Y/data_Whiteley/SOP's/ProteOn SOP.

8.3.8 Open a new method and enter the names of the Antigen and Antibodies to be analyzed.

8.3.9 Follow the manufacturer's instructions to select the run tab and the file name from the pull down. Start the run.

8.4 Data Analysis

8.4.1 X and Y transform the data: shift the data so that the injection start time is at zero and the response levels start at zero.

8.4.2 Non specific binding to the reference channel is subtracted from active surfaces (i.e. subtracting the interspot reference and the blank injection).

8.4.3 The buffer response is subtracted from the active surfaces (double referencing).

8.4.4 The pairing activity is observed when the antibody binds to the captured antigen.

8.4.5 All the ProteOn data are saved on a server with regular backup procedures in operation.

9. REFERENCED DOCUMENTS

9.1. ProteOn Software Instructions for use

9.2. ProteOn Operator's manual

9.3. ProteOn protocols and data are available upon request