

## STANDARD OPERATING PROCEDURE

**Title: Preparation and Analysis of Purified Antigen to their Cognate Purified monoclonal Antibodies by Surface Plasmon Resonance Spectroscopy**

**SOP#: M-122**

**Version #: 1**

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### 1. PURPOSE

This procedure will describe how to measure the binding kinetics of purified antigens and their purified cognate monoclonal antibodies by Surface Plasmon Resonance (SPR) Spectroscopy using a Biacore T100 instrument.

### 2. SCOPE

This procedure applies to all samples that are collected for SPR Spectroscopy analysis using a Biacore T100 instrument. This procedure will describe sample preparation and running the Biacore T100.

### 3. RESPONSIBILITIES

It is the responsibility of the person(s) preparing these reagents to be familiar with lab safety procedures and to have basic laboratory skills. It is the responsibility of the analyst to follow the procedure steps as written and to document any deviations, problems and observations during an experiment on their notebook.

### 4. EQUIPMENT

- Biacore T100, Uppsala Sweden
- Vortex Genie 2 (Fisher, Cat. #12-812)
- Eppendorf Mini Centrifuge Model 5154c
- Biacore Maintenance kit (Cat. #BR1006-51)
- Biacore Series S sensor CM5 chip (Cat. #1005-30)
- Mettler AJ100 Analytical Balance
- Rainin Pipet plus LTS Pipettor, adjustable 100-1000  $\mu$ L

- Rainin Pipet plus LTS Pipettor, adjustable 20-200  $\mu$ L
- Rainin Pipet plus LTS Pipettor, adjustable 2-20  $\mu$ L
- Rainin Pipet plus LTS Pipettor, adjustable 0.1-2  $\mu$ L

## 5. **MATERIALS**

- Polypropylene microtiter u bottom 96 well plates (Greiner Bio One, Cat. #650201)
- 96 well microplate foils (Biacore , Cat. #BR1005-78)
- 7mm plastic vials (Biacore, Cat. #BR1002-12)
- Rubber Caps type 3 (Biacore, Cat. #BR1005-02)
- 4mL vials (SUN-SRi , Cat. #500-160)
- Rubber caps type 5 (Biacore, Cat. #1006-55)
- Amine coupling kit type 2 (Biacore, Cat. #1006-33)
- Acetate Buffer pH 4.5 (Biacore, Cat. #1003-50)
- 1M Hepes Buffer (Invitrogen/Gibco Cat. #15630)
- 5M Sodium Chloride (Quality Biological, Inc. Cat. #351-036-101)
- Phosphoric Acid (Sigma Aldrich, Cat. #215104100G)
- Hydrochloric Acid (Sigma Aldrich, Cat. #H7020)
- 10% SDS, Sodium Dodecyl Sulfate (Gibco, Cat. #15553-035)
- Weighing Paper, 3" x 3" VWR 12577-121 or equivalent
- Falcon BLUE MAX Jr. 15 ml Polypropylene Conical Tube
- Falcon BLUE MAX Jr. 50 ml Polypropylene Conical Tube
- Rainin Space Saver LTS Reloads Pipette Tips. 1000  $\mu$ L (Ranin, Cat. #GPS-L10)
- Rainin Space Saver LTS Reloads Pipette Tips. 250 $\mu$ L (Ranin, Cat. #GPS-L250)
- Rainin Space Saver LTS Reloads Pipette Tips. 20  $\mu$ L (Ranin, Cat. #GPSL-1000)
- Filter Unit 0.20 micron Micrometer porosity 500 mL (Nalgene, Cat. # 450-0020)
- Filter Unit 0.22 micron Micrometer porosity, (Corning, Cat. #Cos430767)
- Eppendorf Centrifuge Tubes, 1.5 ml VWR 20901-551 or equivalent

- Polystyrene 10 mL pipettes, (FALCON, Cat. #356551)
- Polystyrene 25 mL pipettes (Diagger, Cat. number #1BSPS25)

## 6. REAGENTS

- Antigen for desired cancer related protein
- Monoclonal Antibody corresponding to desired cancer related protein
- Recombinant Human Thyroid Stimulating Hormone (TSH) (Seradyn, Inc, Cat. No.# ABT0315).
- Monoclonal Anti-Thyroid Stimulating Hormone ( $\alpha$ -TSH) (Seradyn, Inc., Cat. # MIT0412B)
- Affinipure Rabbit Anti-mouse IgG FC fragment Specific 1.5mg (Jackson Immuno Research, Cat. #315-005-046)
- Surfactant –Amps 20 tween 20 detergent 10% solution (Thermo Scientific Cat. #28320)
- Albumin, Bovine fraction V (Sigma Cat. #A2153)

## 7. PROCEDURE

### 7.1. Preparation of samples for SPR

7.1.1. **Take the Series S CM5 chip out of the refrigerator to warm to room temperature**

7.1.2. **Prepare chip for preconditioning**

7.1.2.1. In separate vials aliquot 4 ml of 10mM HCl, 50mM NaOH, 0.1% SDS and 100mM H<sub>3</sub>PO<sub>4</sub> and cover each vial with type 5 rubber caps

Check the status of the system. If a chip is docked, undock it. Locate the chip icon in the menu bar at top of screen. Choose Eject Chip. Wait until the chip door opens and replace the old chip with the new Series S CM5 chip. Close chip door and identify the chip you are docking.

7.1.2.2. Open preconditioning method (Reference 8.3)

7.1.2.3. Under the general settings tab, set the data collection rate to 10Hz, set the detection to multi, set the sample compartment to 25 °C.

7.1.2.4. Under the assay Steps, add the following four steps: 10mM HCl, 50mM NaOH, 0.1% SDS and 100mM H<sub>3</sub>PO<sub>4</sub>.

- 7.1.2.5. Under the Cycle types on the left hand side under the command insert “sample”.
- 7.1.2.6. On the right hand side add a preconditioning step with a high performance injection type, a contact time of 120 seconds, a dissociation time of 30 seconds, a flow rate of 10 $\mu$ l/min, and it should go over Flow paths 1,2,3,4.
- 7.1.2.7. Put 500 mls of deionized water on both the left and right hand side of the T100 in the plastic filter bottle.
- 7.1.2.8. Click the set up run button
- 7.1.2.9. Run over flow path 1,2,3,4 click “next”
- 7.1.2.10. Click “next” until you see the positions of the preconditioning samples.
- 7.1.2.11. Using reagent rack 2 put the samples in the required positions click “next”
- 7.1.2.12. Go to menu and save method with a unique file name including the run date.
- 7.1.2.13. Read the Prepare Run Protocol list to make sure that all has been completed
- 7.1.2.14. Click Start
- 7.1.2.15. Save data in the designated folder.

### **7.1.3. RAMc chip build**

- 7.1.3.1. Remove Amine coupling kit type 2 from the refrigerator and warm to room temperature.
- 7.1.3.2. Remove (1-Ethyl-3-(3dimethylaminoprpyl) carbodiimide hydrochloride (EDC) and N-Hydroxysuccinimide (NHS)
- 7.1.3.3. Add 10 ml of filtered deionized water to original glass bottle. Cap bottle tightly and agitate until the powder is dissolved
- 7.1.3.4. Label 30 to 40, 7mm vials with the designation EDC. Aliquot 200  $\mu$ l of the EDC solution into the vials cover with type 3 rubber caps and put into a freezer box and store at -20 $^{\circ}$ C. Keep two vials out for the evaluation.
- 7.1.3.5. Repeat 7.1.3.4 for NHS solution. Label the vials appropriately (e.g. NHS) avoid confusion with the EDC vials.
- 7.1.3.6. Place in an ice bucket the two sets of vials (EDC and NHS).
- 7.1.3.7. Remove Rabbit anti mouse IgG from the refrigerator.
- 7.1.3.8. In a 1.5ml eppendorf tube make a 30 $\mu$ g/ml solution of RAMc.

- 7.1.3.9. Add 490  $\mu$ l of 10mM sodium acetate buffer pH 4.5 and to eppendorf and 10  $\mu$ l of RAMc, cover with type 3 rubber caps
- 7.1.3.10. Vortex and aliquot 250  $\mu$ l into two tubes marked RAMc, cover with type 3 rubber caps
- 7.1.3.11. Add 150  $\mu$ l of Ethanolamine to two 7mm tubes marked ethanolamine, cover with type 3 rubber caps.
- 7.1.3.12. Make 1M HCL by adding 1.25 ml of Hydrochloric Acid to 15.6ml of water
- 7.1.3.13. To make 100mM HCL add 12.5 ml of 1M HCL to 112.5ml of water
- 7.1.3.14. Add 75  $\mu$ l of 100mM HCL to two 7mm tubes and cover with type 3 rubber caps
- 7.1.3.15. Cover two empty 7mm tubes with type 3 rubber caps and set aside.
- 7.1.3.16. Open the generic wizard to Build a RAMc chip
- 7.1.3.17. Choose chip type CM5 from the pull down Menu.
- 7.1.3.18. Choose to immobilize flow cells one and two by selecting "aim for an immobilized level"
- 7.1.3.19. Choose the Amine coupling method from the pull down menu and write in RAMc as the ligand
- 7.1.3.20. The target level is 8000Ru and the wash solution is 100mM HCL and click next.
- 7.1.3.21. Choose to Prime before the run and click "next"
- 7.1.3.22. Line up the reagents in Sample and Reagent Rack 1 make sure that the reagents are in their proper position and click "next".

#### **7.1.4. Antibody Antigen preparation**

- 7.1.4.1. Remove Antigens and antibodies to be studied.
- 7.1.4.2. Make the Antibody Characterization Buffer (1x HBS (Hepes Buffered Saline), with 0.005% Tween 20 and 0.1% BSA)
  - 7.1.4.2.1. In a 500ml graduated Cylinder add 50 ml of 10xHBS, 500 mg of BSA and 250  $\mu$ l of 10% Tween 20 solution add water to reach final volume of 500ml
- 7.1.4.3. Place parafilm on top of the graduated cylinder and mix well then filter through 500ml filter and degas under vacuum for 15 minutes

- 7.1.4.4. To prepare antibody samples add 198 $\mu$ l of Antibody Characterization Buffer to a 7 mm tube and 2  $\mu$ l of the purified monoclonal antibody (or 60ul of supernatants and 140  $\mu$ l of Antibody Characterization Buffer)
- 7.1.4.5. Determine the Antigen concentration and what dilution factor is needed to make a final concentration of 500nM. (i.e.) if the Antigen is 2.4 mg/ml you then divide by the molecular weight so 25700D Da. Therefore 2.4/25700 is 0.0000093385. or 93  $\mu$ M
- 7.1.4.6. Calculate the number of cycles of antigen that will be needed to be run in the method and multiply by the minimum volume needed. Manually calculate a dilution chart similar to the one that follows

Concentration	dilution factor	previous	buffer	total
1000n	1:93	8.6	791.4	800
500n	1:2	681.2	681.2	1362
250n	1:2	662.5	662.5	1325
125n	1:2	62.5	62.5	1250
62.5n	1:2	500	500	1100
31.25n	1:2	500	350	700

In the above experiment we ran three monoclonal antibodies and three hybridoma supernatants that were being tested in the method so in theory we needed approximately 700  $\mu$ l to account for all dead volumes needed from this dilution.

- 7.1.4.7. Anti-TSH and TSH are being used as the positive controls in these experiments. Dilutions for the positive controls is as follows:

Concentration	dilution factor	previous	buffer	total
1000n	1:3.6	13.8	36.1	50
100n	1:10	40	360	400
50n	1:2	200	200	400
25n	1:2	200	200	400
12.5n	1:2	150	150	300
6.25n	1:2	100	100	200

### 7.1.5. General Method for Kinetic Titration of Antigens

- 7.1.5.1. Open the generic method for the kinetic titration of antigens
- 7.1.5.2. Under the “general settings” tab set the data collection rate to 10Hz, set the detection to dual, set the sample compartment to 25°C.
- 7.1.5.3. Under the “Assay steps” you would need to modify the names of the samples that you intend to run being sure to leave the positive controls at both the beginning and the end. Make sure that the temperature settings remain at 25°C in buffer A, the recurrence button is off and the number of replicates is set to 1 time as entered.
- 7.1.5.4. Under the “Cycle types” on the left hand side under the command select “insert sample”.
- 7.1.5.5. At the top left add a start up, antigen, Antibody and buffer
- 7.1.5.6. On the bottom left be sure to add the command “sample”
- 7.1.5.7. For the start up cycles use the high performance injection type with a contact time of 60 seconds. 60seconds dissociation time, and a flow rate of 30 $\mu$ l/min over both flow cells
- 7.1.5.8. For cycle types antigen and buffer we want to use the single cycle kinetics for the cycle types, with 5 concentrations and a contact time of 120 seconds and 600 seconds dissociation flow rate of 10 $\mu$ l/min and over both flow paths
- 7.1.5.9. A high performance injection for the antibody cycles use a contact time of 120 seconds and a dissociation time of 300 seconds, a flow rate of 10 $\mu$ l/min over ONLY the second flow path.
- 7.1.5.10. The variable setting tab should only list the cycle types on the left hand side and ask you to define all values at run time.
- 7.1.5.11. The verification tab should say: “The method has been verified” and can be used to set up a run.
- 7.1.5.12. Click the set up run tab and click “next”
- 7.1.5.13. Choose the appropriate flow cell to run the experiment on and click “next”
- 7.1.5.14. Click on each assay step and be sure that each variable in the assay step has the correct concentrations. Also make any name changes that are needed as some names

may have been changed in the first tab of the method.  
Finally click “next”.

- 7.1.5.15. Look over the cycle run list and make sure things are in the correct order and click “next”.
- 7.1.5.16. For the system preparations make sure that the “prime before run” and “normalize” buttons are checked, click “next”
- 7.1.5.17. On the rack position page make sure that your samples are in the specified positions on this page, click “next”.
- 7.1.5.18. Prepare to run the protocol, double check each line and be sure to put enough buffer in the correct positions and save the method in the appropriate location.

#### **7.1.6. Processing the data**

- 7.1.6.1. Under the Sensograms tab look at all the data at once
- 7.1.6.2. Choose the Kinetic affinity button at the top of the page. Choose surface bound
- 7.1.6.3. Pick the appropriate antigen binding to the antibody.
- 7.1.6.4. Choose the blank injection which should be the cycle just before the checked cycle on the left hand side and click “next”
- 7.1.6.5. The data will now be FC referenced and buffer subtracted, hence double referenced choose kinetics and click “next”
- 7.1.6.6. Using the 1:1 binding model click fit and once fit click “next”
- 7.1.6.7. The data is then processed and you will be able to see the parameters when you choose the report button  
The rate of association ( $k_a$ ), dissociation ( $k_d$ ), the Equilibrium Constant (KD), the chi square value (the smaller the better) and the U-value uniqueness of the fit will all be calculated.

### **8. REFERENCED DOCUMENTS**

- 8.1. Operation Manual, BIACORE T100
- 8.2. Operation Manual, Mettler AJ100 Analytical Balance
- 8.3. Software Routines: Available from the Antibody Characterization Laboratory, SAIC Frederick
  - 8.3.1. Preconditioning Method for T100 Chips