STANDARD OPERATING PROCEDURE
Title: Kinetics and Affinity Evaluation of Monoclonal Antibodies and Proteins Using the Biacore T200
SOP#: M-140
Version #: 1 Author: T. Caceres
Date Approved: July 15, 2021 Date Modified:

1. PURPOSE

This procedure will describe how to measure the equilibrium constant, K_D, the association rate constant, K_a, and the dissociation rate constant, K_d, of purified proteins or peptides and their purified cognate monoclonal antibodies by Surface Plasmon Resonance (SPR) Spectroscopy using a Biacore T200 instrument.

2. SCOPE

This procedure will describe sample preparation and operation of the Biacore T200 using either a capture or amine coupling multiple cycle method to measure affinity and kinetics parameters. This SOP includes a performance qualification test that can be run to evaluate the performance of the Biacore T200 instrument prior to evaluating test samples.

3. RESPONSIBILITIES

It is the responsibility of the person(s) performing 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 steps as written and to document any deviations, problems, and observations during an experiment in their laboratory notebook.

4. EQUIPMENT

4.1. Biacore T200, Uppsala Sweden, Model #T200, Serial # 2650784

4.2. Fisher Vortex Genie 2

4.3. Eppendorf Mini Centrifuge Model 5154c

4.4. Mettler AJ100 Analytical Balance

4.5. Rainin Pipet plus LTS Pipettor, adjustable 100-1000 µL

4.6. Rainin Pipet plus LTS Pipettor, adjustable 20-200 µL

4.7. Rainin Pipet plus LTS Pipettor, adjustable 2-20 µL

4.8. Rainin Pipet plus LTS Pipettor, adjustable 0.1-2 µL

4.9. Reagent rack Type I (Cytiva rack included with instrument)

4.10. Reagent rack Type II (Cytiva rack included with instrument)

4.11. Combined sample and reagent rack (Cytiva rack included with instrument)

5. <u>MATERIALS</u>

5.1. Polypropylene microtiter u bottom 96 well plates (Greiner Bio One, Cat. #650201)

5.2. Biacore Maintenance kit (Cytiva, Cat# BR1006-51)

5.3. Biacore Series S sensor CM5 chip (Cytiva, Cat# BR100530)

5.4. Series S sensor chip Protein A (Cytiva, Cat# 29127555)

5.5. 96 well microplate foils (Cytiva, Cat. #BR1005-78)

5.6. 7 mm (0.8 mL) plastic vials (Cytiva, Cat. #BR1002-12)

5.7. Penetrable Kraton™ G (SEBS) caps type 3 for 7 mm vials(Cytiva, Cat. #BR1005-02)

5.8. 15 mm (4.0 mL) vials (Cytiva, Cat. # 29266981)

5.9. Penetrable Kraton™ G (SEBS) caps type 5 for 15 mm vials (Cytiva, Cat. #BR1006-55)

5.10. Getting Started T200 kit: (Cytiva, Cat#. 28980886)

5.11. Amine coupling kit (Cytiva, Cat. #BR-1000-50)

5.12. Sodium Acetate Buffer pH 5.0 (Cytiva, Cat. #1003-50)

5.13. 10X HBS-EP+ buffer (Cytiva, Cat#. BR100826)

5.14. 5M Sodium Chloride (Quality Biological, Inc. Cat. #351-036-101)

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

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

5.17. Rainin Space Saver LTS Reloads Pipette Tips. 1000 µL (Ranin, Cat. #GPS-L10)

5.18. Rainin Space Saver LTS Reloads Pipette Tips. 250µL (Ranin, Cat. #GPS-L250)

5.19. Rainin Space Saver LTS Reloads Pipette Tips. 20 µL (Ranin, Cat.

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#GPSL-1000)

5.20. Filter Unit 0.20 µm porosity 500 mL (Nalgene, Cat. # 450-0020)

5.21. Filter Unit 0.22 µm porosity, (Corning, Cat. #Cos430767)

5.22. Eppendorf Centrifuge Tubes, 1.5 ml (VWR, Cat. #20901-551 or equivalent)

5.23. Polystyrene 10 mL pipettes, (FALCON, Cat. #356551)

5.24. Polystyrene 25 mL pipettes (FALCON, Cat. number #1BSPS25)

5.25. Surfactant –Amps 20 Tween 20 detergent 10% solution (Thermo Scientific Cat. #28320)

5.26. H_2O, ultrapure deionized and 0.22 uM filtered

5.27. 10 mM Glycine pH 1.5 (Cytiva, Cat # BR100354).

6. REAGENTS

6.1. Proteins or peptides corresponding to desired target

6.2. Monoclonal antibody or antibodies corresponding to desired cancer related protein or peptide

7. PROCEDURE

7.1. Preparation of Biacore T200 instrument

7.1.1. Switch on the instrument. The lamps on the front panel should light in the following sequence: a) All the lamps light for a few seconds and then go out. b) The green Ready lamp lights. c) The yellow Temperature lamp flashes to indicate non-stabilized temperature, and then is steadily lit when the temperature at the detection unit is stable. The time required for temperature stabilization depends on the set temperature and ambient temperature. The instrument uses the last analysis and sample compartment temperatures set before shutdown as default. Temperature stabilization should not take more than an hour.

7.1.2. Open the right pump compartment door and check that the clamp of the peristaltic pump is properly fastened: the lever should be in a vertical position.

7.1.3. Place a 2-liter bottle for waste solution on the waste and water tray. Fit the cap carrying the waste tubes on to the bottle. Tighten the tube fittings by hand. Always use a 2-liter bottle for waste. Fill a 500 ml bottle with ultrapure deionized, 0.22 µm filtered water. Fit a cap with gasket and place it on the waste bottle and into the water tray. Insert the water tube into the water bottle.

7.1.4. Prepare fresh 500 mL of 1X HBS-EP+ buffer by diluting 50 mL of 10X buffer into 450 mL of ultrapure deionized, 0.22 µm filtered water in a 500 mL glass bottle. Mix well.

7.1.5. Prepare the instrument by inserting Tube A (from the instrument) into the 500 mL of fresh 1x HBS-EP+. Make sure that the tube reaches to the bottom of this buffer bottle.

7.2. Preparation for Instrument Performance Qualification

Note: The Performance Qualification should be run at least one time per month to evaluate the T200 performance.

7.2.1. Preparation of control protein

7.2.1.1. Take the Getting Started T200 kit out of the refrigerator to warm to room temperature. **The kit components should be used within one week of opening (Date the box after opening).** Remove the anti-b2micro antibody from the kit. Dilute 1 uL of the antibody (1 mg/mL) into 1 mL of 1X HBS-EP+ buffer for a final concentration of 1 ug/mL. Transfer 600 uL of diluted antibody (ligand) to a 0.8 mL (7 mm) polypropylene micro-vial and seal with a cap. Label the vial appropriately.

7.2.1.2. Start Biacore T200 Control Software by clicking on the Start menu and select Biacore T200 Control Software from the Biacore menu. The software establishes connection with the instrument, which takes about 30 seconds.

7.2.1.3. Undock the maintenance sensor chip (obtained from the Cytiva Maintenance Kit) and store it in a 50 mL conical tube to keep it free of dust.

7.2.1.4. Insert a Series S Protein A sensor chip into the machine and select Protein A in the description. Keep record of the number of times the chip has been used. Discard the chip after 800 cycles or after six months, whichever comes first.

7.2.1.5. Prime the instrument with 1X HBS-EP+ buffer by selecting Tools -> Prime. Follow the instructions in the dialog boxes and start the procedure. When the procedure is finished (after about 7 minutes), click on Close. A continuous standby flow of running buffer is started automatically when the procedure is finished.

7.2.1.6. Prepare the b2micro analyte (control protein) as follows. In a 1.5 mL eppendorf tube, dilute 3.76 uL of b2micro (8.5 uM) into 496.24 uL of 1X HBS-EP+ buffer for a final concentration of 64 nM. Dilute 100 uL of 64 nM into 300 uL buffer to prepare a 4-fold dilution. Proceed with additional 4-fold dilutions down to 0.25 nM.

7.2.2.
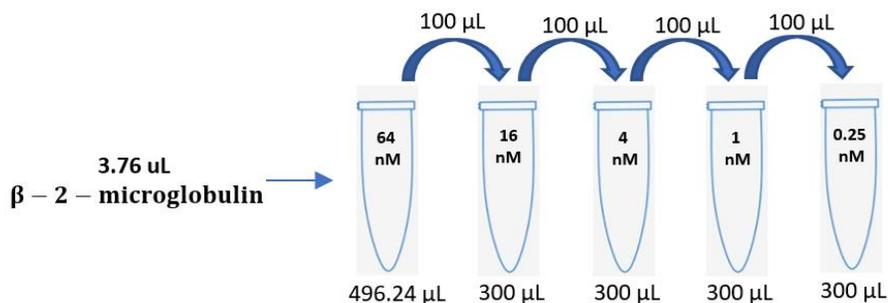
Open the Biacore wizard file **PQ_Wizard_01192021** that is located in the path Share -> Instrumentation -> Biacore T200 -> Protocols. The wizard should open showing the *Injection Sequence* screen.

7.2.3. Verify the assay steps and settings:

7.2.3.1. Injection sequence:

- Detection flow path: 2-1.
- Chip type: Protein A.
- Ligand Capture is checked.
- Regeneration is checked with 1 cycle.

7.2.3.2. Setup:

- Startup: Check run startup cycles is checked
- Solution: 1X HBS Buffer
- Number of cycles: 3

7.2.3.3. Injection parameters:

- Ligand capture:
  - ❖ Ligand: Anti-2bmicro
  - ❖ Contact time: 60 (s)
  - ❖ Flow rate: 10 (uL/min)
  - ❖ Stabilization period: 0 (s)
- Sample:
  - ❖ Contact time: 120 (s)
  - ❖ Flow rate: 50 (uL/min)
  - ❖ Dissociation time: 180 (s)

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- Regeneration:
    - ❖ Solution: 10 mM Glycine pH 1.5
    - ❖ Contact time: 30 (s)
    - ❖ Flow rate: 50 (uL/min)
    - ❖ Stabilization period: 0 (s)

7.2.3.4. Samples:

- Verify that the samples prepared match according to the table seen below.

	Sample id	MW (Da)	Concentration nM	Concentration µg/ml
1	b2micro	12000	0	0.000
2	b2micro	12000	0.25	0.003000
3	b2micro	12000	1	0.01200
4	b2micro	12000	4	0.04800
5	b2micro	12000	16	0.1920
6	b2micro	12000	64	0.7680
7	b2micro	12000	64	0.7680
8				

Run order: ⦿ As entered   ◯ Increasing concentration

Help   Import...   Control Samples...   < Back   Next >   Close

7.2.3.5. System Preparations:

- Check prime before run
- Temperature settings:
    - Analysis temperature – 25C
    - Sample compartment temperature – 25C

7.2.3.6. Rack positions: The software will calculate the minimum required volumes for each sample as seen in the table below. Transfer at least the minimum required volumes of samples and buffers to 15 mM and 7 mm vials and seal with

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ventilated kraton G (SEBS) rubber, penetrable caps. Verify that there are no bubbles in the vials. Place the vials in the following positions on rack as seen below.

Position	Volume (µl)	Content	Type	Sample 1 Conc (nM)	Sample 1 MW (Da)
R1 A1	158	b2micro	Sample	0	12000
R1 A2	158	b2micro	Sample	0.25	12000
R1 A3	158	b2micro	Sample	1	12000
R1 A4	158	b2micro	Sample	4	12000
R1 A5	158	b2micro	Sample	16	12000
R1 A6	158	b2micro	Sample	64	12000
R1 A7	158	b2micro	Sample	64	12000
R1 B1	158	Buffer	Startup		
R1 B2	158	Buffer	Startup		
R1 B3	158	Buffer	Startup		
R2 A1	970	Glycine pH 1.5	Regeneration		
R2 B1	290	Anti-2bmicro	Capture		

7.2.4. Click on next.

7.2.5. Complete Run Protocol steps.

7.2.6. Start the Run.

7.2.7. Document the run start time and estimated run time. The assay should run for approximately two hours.

7.2.8. When the run is complete, perform the data analysis as described in section 7.5. Save the results from the Performance Qualification (PQ) to the Shared Drive and document the run appropriately in a laboratory notebook.

7.2.9. If the Performance Qualification passes, proceed to analyze test samples using the same Series S Protein A sensor chip using a Capture Run in step 7.3 or using a new Series S CM5 sensor chip using Amine Coupling in step 7.4. Place the instrument in stand-by with 1X HBS buffer while preparing the test samples.

7.2.10. If this is the last experiment to be performed, eject the Series S Protein A sensor chip. Take the chip insert out of the cassette and submerge the chip insert in HBS-EP+ buffer in a 50 mL conical tube and store at 4°C. Insert the Maintenance chip and run a desorb operation. After the desorb operation, the instrument will automatically start standby mode. Leave the system running in ultrapure deionized H_2O. The Protein A chip can be used multiple times. To re-use the chip, remove the chip insert from storage, rinse with ultrapure deionized H2O, and dry. Then put the chip insert back into the cassette. Discard the chip after 800 cycles or after six months, whichever comes first.

7.3. Preparation for Capture Run Using a Series S Protein A Sensor Chip

Note: The following procedure could also be applied for the use of the Series S Protein G Sensor Chip. In this case, select "Custom" instead of selecting "Protein A" as chip type in the software.

7.3.1. Check buffer volumes and empty waste container.

7.3.2. Use the same Series S Protein A sensor chip that was used in the QC.

7.3.3. Prime the instrument with 1X HBS-EP+ buffer by selecting Tools -> Prime. Follow the instructions in the dialog boxes and start the procedure. When the procedure is finished (after about 7 minutes), click on Close. A continuous standby flow of running buffer is started automatically when the procedure is finished.

7.3.4. Preparation of the test sample.

7.3.4.1. Remove the test sample antibody. Dilute the antibody into 1 mL of 1X HBS-EP+ buffer to the optimum concentration that was determined in assay development tests or elsewhere. ***Please refer to Appendix A for procedures to optimize ligand concentration.*** Commonly used concentrations usually fall in the 5 – 25 ug/mL range. Transfer 600 uL of diluted antibody to a 0.8 mL polypropylene micro-vial and seal with a cap.

7.3.4.2. Prepare the test protein or peptide analyte at a concentration range that is approximately 0.1X – 100X the K_D of the binding interaction. If no knowledge of the kinetics is available, dilute test protein or peptide into 500 uL of 1X HBS-EP+ buffer for a final concentration of 1024 nM. Prepare 4-fold dilutions down to 1 nM as seen below.
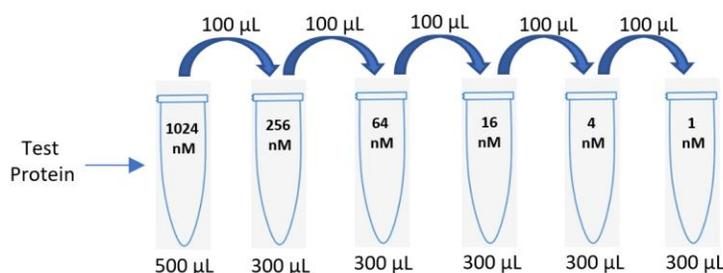
7.3.5. Open the Biacore method file **T200_SOP_Method_Capture** that is located in the path Share -> Instrumentation -> Biacore T200 -> Protocols. The method should open showing the *Overview* screen.

7.3.6. Click on *General Settings* and verify the following settings:

7.3.6.1. At start:

- Data collection rate: 10 Hz
- Detection: Dual
- Sample compartment temperature: 25°C

7.3.6.2. Miscellaneous settings:

- Concentration unit: nM

7.3.7. Click on *Assay Steps.*

    7.3.7.1. Click on *Startup* in the middle of the panel.

- Assay step properties:
  - Base Settings:
    - ❖ Name: Startup
    - ❖ Purpose: Startup
    - ❖ Connect to cycle type: Kinetics
  - Assay step preparations:
  - ❖ Temperature: 25C
  - ❖ Buffer: A
  - Number of replicates:
    - ❖ Times: 3
    - ❖ As entered: 1,2,3,1,2,3

    7.3.7.2. Click on *Sample* in the middle of the panel.

- Assay step properties:
  - Base Settings:
    - ❖ Name: Sample
    - ❖ Purpose: Sample
    - ❖ Connect to cycle type: Kinetics
  - Assay step preparations:
    - ❖ Temperature: 25C
    - ❖ Buffer: A
  - Number of replicates:
    - ❖ Times: 1
    - ❖ As entered: 1,2,3,1,2,3

7.3.8. Click on *Cycle Types*

    7.3.8.1. Click on the Commands tab and verify the following settings:

- Capture 1:
  - Settings for Capture 1:
    - ❖ Capture solution: Enter test antibody name
    - ❖ Contact time: 60 seconds

- ❖ Flow rate: 10 uL/min
- ❖ Flow path: Second
- Sample 1:
  - ▪ Settings for Sample 1:
    - ❖ Type: High Performance
    - ❖ Sample solution: Is Variable
    - ❖ Contact time: 120 (s)
    - ❖ Dissociation time: 300 (s)
    - ❖ Flow rate: 50 (uL/min)
    - ❖ Flow path: Both
- Regeneration 1:
  - ▪ Settings for Regeneration 1:
    - ❖ Regeneration Solution: 10 mM Glycine pH 1.5
    - ❖ Contact time: 30 (s)
    - ❖ Flow rate: 50 (uL/min)
  - ▪ Flow path: Both

7.3.9. Click on the *Variable Settings*.

    7.3.9.1.   Verify the following assay steps:

- Startup: Define all values in method
- Sample: Define all values at run time

7.3.10. Click on *Setup Run*.

    7.3.10.1. Verify the Detection:

- Flow path: 2-1
- Click next

7.3.11. Edit the *Variables* Table accordingly. The listed concentrations should match the concentrations that were prepared in 7.3.4.2.

    7.3.11.1.   Input the name of the test protein in the sample solution column.

    7.3.11.2.   Input the molecular weight of the test protein in the MW (Da) column.

    Note: Always begin and end the titration with 0

nM. Always repeat one concentration.

7.3.11.3. Click next

7.3.12. Check _Prime before run_ under the System Preparations and click Next. Do **NOT** check Normalize.

7.3.13. The software will calculate the minimum required volumes for each sample as seen in the table below. Transfer at least the minimum required volumes of samples and buffers to appropriate vials and seal with ventilated kraton G (SEBS) rubber, penetrable caps. Verify that there are no bubbles in the vials. Place the vials in the following positions on rack as seen below.

Position	Volume (µl)	Content	Type	Sample 1 Conc (nM)	Sample 1 MW (Da)
R1 A1	306	test antigen	Sample	0	
R1 A2	158	test antigen	Sample	1	
R1 A3	158	test antigen	Sample	4	
R1 A4	158	test antigen	Sample	16	
R1 A5	158	test antigen	Sample	64	
R1 A6	158	test antigen	Sample	256	
R1 A7	306	test antigen	Sample	1024	
R1 B1	454	Buffer	Startup		
R1 D1	356	Test Antibody	Capture		
R1 F1	1084	Glycine pH 1.5	Regeneration		

7.3.14. Click on next.

7.3.15. Complete Run Protocol steps.

7.3.16. Start the Run.

7.3.17. Document the run start time and estimated run time.

7.3.18. When the run is complete, perform the data analysis as described in section 7.5.

7.3.19. If additional experiments will be run using the antibody ligand and protein analyte pair, place the instrument in stand-by with 1X HBS buffer

7.3.20. If this is the last experiment to be performed, eject the Series S Protein A sensor chip. Store the chip as described in 7.2.10. The Protein A chip can be used multiple times. Discard the chip after 800 cycles or after six months, whichever comes first.

7.3.21. Insert the Maintenance chip and run a desorb operation. Leave the system running in ultrapure deionized, 0.22 µm filtered water. Prior to re-use, rinse the chip with deionized water and carefully dry the raised glass side. Slide the chip insert back into the cassette. Discard the chip after 800 cycles or after six months, whichever comes first.

7.4. Preparation for an Amine Coupling Immobilization Run Using a Series S CM5 Chip

Note: It is recommended to use amine coupling if it is known that the interaction between the antibody and test analyte shows very tight binding (low pM binding).

7.4.1. Check buffer volumes and empty waste container.

7.4.2. Preparation for covalent immobilization.

   7.4.2.1 Remove the Amine Coupling Kit from 2 - 8°C. If the kit has already been prepared, skip to 7.4.3. The kit contains:

      7.4.2.1.1.   N-Hydroxysuccinimide (NHS),115 mg

7.4.2.1.2. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), 750 mg

7.4.2.1.3. 1M Ethanolaminehydrochloride-NaOH pH 8.5, 10.5 mL

7.4.2.2. Dissolve the EDC and NHS by adding 10.0 mL of filtered, deionized water to each vial.

7.4.2.3. Cap vials tightly and vortex until the solids are completely dissolved.

7.4.2.4. Prepare separate 500 uL aliquots of the EDC and NHS solutions for storage.

7.4.2.5. Store the aliquots of EDC and NHS frozen at -18°C or below. Ethanolamine may be stored at 2°C to 8°C.

7.4.3. Undock the maintenance sensor chip or the Protein A chip from the PQ and store it in a 50 mL conical tube as described in 7.2.10.

7.4.4. Insert a new Series S CM5 chip into the machine and select CM5 in the description. Each CM5 chip has 4 flow cells. After an antibody is immobilized via covalent bonding to one flow cell, it cannot be removed. Therefore, one CM5 chip could potentially have three different antibodies immobilized on it (Flow cell one must always be used as a reference). If only flow cells one (Fc1) and two (Fc2) are utilized in an assay, flow cells three and four (Fc3 and Fc4) on the same chip could be used to immobilize two new antibodies in a separate assay.

7.4.5. Prime the instrument with 1X HBS-EP+ buffer by selecting Tools -> Prime. Follow the instructions in the dialog boxes and start the procedure. When the procedure is finished (after about 7 minutes), click on Close. A continuous standby flow of running buffer is started automatically when the procedure is finished.

7.4.6. Prepare the following for the immobilization of two flow cell surfaces: EDC (500 uL), NHS (500 uL), two blank tubes for mixing EDC/NHS and ethanolamine (1.0 mL).

7.4.7. Remove the test sample antibody. Dilute the antibody into 1 mL of the pre-conditioning buffer 10 mM sodium acetate buffer, pH 5.0, to the optimum antibody concentration that was determined in assay development tests. Commonly used concentrations usually fall in the 5 – 25 ug/mL range. Mix well. Transfer 200 uL of diluted antibody to a 0.8 mL polypropylene micro-vial and seal with a cap. ***See Appendix A for procedures to optimize pre-concentration pH and ligand concentration.***

7.4.8. Within the Biacore T200 Control software, select *File* and *Open/New Wizard Template.* Browse to the **SOP_Method_Amine_Coupling_Immobilization_01152021** template located within the Shared Drive.

Share -> Instrumentation -> Biacore T200 -> Protocols

7.3.9. Verify the Chip Type – CM5 and flow cells per cycle – 1.

7.4.10. Check boxes for Flow cell 1 (Fc 1) and Flow cell 2 (Fc 2). Select 'Blank immobilization" for Fc 1. For Fc 2, type the name of the test antibody for the ligand name.

    7.4.10.1. Select Aim for Immobilized Level if you don't know the contact time.

- Calculate the target level response units (RU) using the following formula:

$$R_L = (R_{max} * MW_L)/(MR_A * Valency_L)$$

Where $R_{max} = 30$

- Use 50 nM NaOH for the wash solution.

    7.4.10.2. Select Specify Contact time and Flow rate when following a protocol that specifies the contact time

    7.4.10.3. Click Next.

7.4.11. Check the box for Prime.. Do not check box for Normalize. Leave temperature settings as they are. Click Next.

7.4.12. The wizard will calculate the minimum required volumes and indicate the vial positions. Place your reagents, ensuring adequate volumes, in the appropriate positions and click next. Don't forget the empty tubes for mixing the EDC/NHS. Note the estimated run time (approximately 1.0 hour).

SOP_Method_Amine_Coupling_Immobilization_01152021 - Rack Positions

Reagent Rack 2

Position	Volume (µl)	Content	Type
R2 B1	89	EDC	Immob Fc 1
R2 B2	89	NHS	Immob Fc 1
R2 B3	Empty	EDC/NHS, min. capacity 124µl	Immob Fc 1
R2 B4	129	Ethanolamine	Immob Fc 1
R2 C1	89	EDC	Immob Fc 2
R2 C2	89	NHS	Immob Fc 2
R2 C3	Empty	EDC/NHS, min. capacity 124µl	Immob Fc 2
R2 C4	129	Ethanolamine	Immob Fc 2
R2 C5	98	Test Antibody	Immob Fc 2

None

Help | Menu ▼ | Eject Rack | < Back | Next > | Close

7.4.13. Save this immobilization file before running this wizard to the appropriate project folder located in the Biacore T200 instrument folder on the Shared Drive.

7.4.14. Start the immobilization.

7.4.15. Remove the test protein or peptide analyte from 4°C, -30°C, or -80°C

7.4.16. Dilute the protein or peptide test analyte to a concentration range that is approximately 0.1X – 100X the K_D of the binding interaction. If no knowledge of the kinetics is available, dilute

test protein into 500 uL of 1X HBS-EP+ buffer for a final concentration of 1024 nM. Prepare 4-fold dilutions down to 1 nM as seen below.
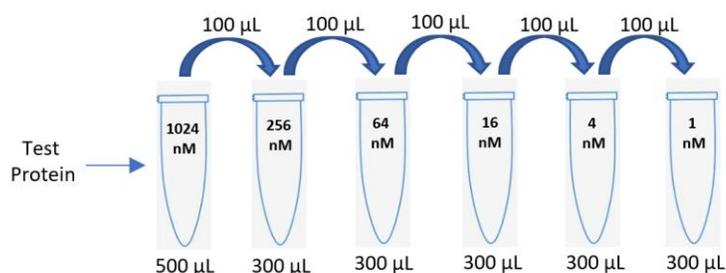
7.4.17. When the immobilization is finished, open the **T200_SOP_Direct_Kinetics** method. The method should open with the *Overview* screen.

7.4.18. Click on *General Settings* and verify the following settings:

    7.4.18.1. At start:

- Data collection rate: 10 Hz
- Detection: Dual
- Sample compartment temperature: 25°C

    7.4.18.2. Miscellaneous settings:

- Concentration unit: nM

7.4.19. Click on Assay Steps.

    7.4.19.1. Click on *Startup* in the middle of the panel.

- Assay step properties:
  - Base Settings:
    - ❖ Name: Startup
    - ❖ Purpose: Startup
    - ❖ Connect to cycle type: Kinetics
  - Assay step preparations:
    - ❖ Temperature: 25C

- ❖ Buffer: A
  - ▪ Number of replicates:
    - ❖ Times: 3
    - ❖ As entered: 1,2,3,1,2,3

7.4.19.2. Click on *Sample* in the middle of the panel.

- • Assay step properties:
  - ▪ Base Settings:
    - ❖ Name: Sample
    - ❖ Purpose: Sample
    - ❖ Connect to cycle type: Kinetics
  - ▪ Assay step preparations:
    - ❖ Temperature: 25C
    - ❖ Buffer: A
  - ▪ Number of replicates:
    - ❖ Times: 1
    - ❖ As entered: 1,2,3,1,2,3

7.4.20. Click on *Cycle Types*

7.6.1.1. Click on the Commands tab and verify the following settings:

- • Sample 1:
  - ▪ Settings for Sample 1:
    - ❖ Type: High Performance
    - ❖ Sample solution: Is Variable
    - ❖ Contact time: 120 (s)
    - ❖ Dissociation time: 300 (s)
    - ❖ Flow rate: 50 (uL/min)
    - ❖ Flow path: Both
- • Regeneration 1:
  - ▪ Settings for Regeneration 1:

❖ Regeneration Solution: 10 mM Glycine pH 1.5

❖ Contact time: 30 (s)

❖ Flow rate: 50 (uL/min)

▪ Flow path: Both

7.4.21. Click on *Setup Run*.

7.4.21.1. Select the Detection flowpath: 2-1. Click Next.

7.4.22. Edit the Variables Table according to the name of the test protein or peptide analyte:

7.4.22.1. Input the name of the test protein or peptide analyte. Input the molecular weight of the test protein or peptide analyte in the MW (Da) column. The listed concentrations should match the concentrations that were prepared in 7.4.16. **Note: Always begin and end the titration with 0 nM. Always repeat one concentration.**

7.4.22.2. Click next.

7.4.23. Check *Prime before run* under the System Preparations and click Next. Do **NOT** check Normalize.

7.4.24. The software will calculate the minimum required volumes for each sample as seen in the table below. Transfer at least the minimum required volumes of samples and buffers to appropriate vials and seal with ventilated kraton G (SEBS) rubber, penetrable caps. Verify that there are no bubbles in the vials. Place the vials in the following positions on rack as seen below.

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T200_SOP_Direct_Kinetics - Rack Positions

Reagent Rack 2

96 Well Microplate

Position	Volume (µl)	Content	Type	Sample 1 Conc (nM)	Sample 1 MW (Da)
R1 A1	158	Test Analyte	Sample	0	
R1 A2	158	Test Analyte	Sample	1	
R1 A3	158	Test Analyte	Sample	4	
R1 A4	158	Test Analyte	Sample	16	
R1 A5	158	Test Analyte	Sample	64	
R1 A6	158	Test Analyte	Sample	256	
R1 A7	158	Test Analyte	Sample	1024	
R1 A8	158	Test Analyte	Sample	1024	
R1 A9	158	Test Analyte	Sample	0	
R1 B1	158	Buffer	Startup		
R1 B2	158	Buffer	Startup		
R1 B3	158	Buffer	Startup		
R2 A1	1084	Regen Buffer	Regeneration		

Help   Menu ▼   Eject Rack   < Back   Next >   Close

7.4.25. Click on next.

7.4.26. Complete the *Prepare Run Protocol* steps.

7.4.27. Start the Run.

7.4.28. Document the run start time and estimated run time.

7.4.29. When the run is complete, perform the data analysis as described in section 7.5.

7.4.30. If additional experiments will be run using the antibody ligand and protein antigen pair, place the instrument in stand-by with 1X HBS buffer.

7.4.31. If this is the last experiment to be performed, eject the Series S CM5 chip. Label a 50 mL conical tube with the antibody/protein/peptide pair and the project name. Store the chip as described in 7.3.21. Insert the Maintenance chip and run a desorb operation. Leave the system running in deionized, 0.22

um filtered water. Shut down the instrument if it will be
unattended for more than two weeks.

7.5. Data Analysis

7.5.1. The Evaluation software should open automatically at the end of
any run with the current data loaded. If data is to be analyzed
later, open the Biacore T200 Evaluation Software 3.2. Select
File -> Open and browse to the appropriate project folder where
the run was saved.

7.5.2. Look across the top menu toward the right side and select
Screening, Kinetic screen.

7.5.3. Multiple result files can be included in one analysis. For the PQ
data analysis, there is one result file. Click Next.

7.5.4. Thumbnail(s) will appear on the left, and the detailed
sensorgram with reference subtracted and blank subtracted
sample responses appears on the right. The 0 nM concentration
sample has been subtracted from the other curves. View the
blank.

7.5.5. Above the thumbnail, choose View, Extended thumbnails.

7.5.6. Above the thumbnail, click on Settings, Fit Settings. Choose the
1:1 model. Click ok. Now click Fit.

7.5.7. Choose the blank injection which is be the cycle just before the
checked cycle on the left-hand side and click Next.

7.5.8. The data will now be flow cell referenced and buffer subtracted,
hence double referenced. Choose kinetics and click Next.

7.5.9. Using the 1:1 binding model, click Fit. Click Next.

7.5.10. The data is then iteratively processed.

7.5.11. Examine the Quality Control results. All QC results should pass.

7.5.12. Examine the Report results. The association rate constant (k_a),
the dissociation rate constant (k_d), the equilibrium constant (K_D),
the R_{max}, and the Chi^2 will all be calculated.

7.5.13. For the PQ to pass, the K_D must be 1 nM +/- 0.2.

7.5.14. The R_{max} must be < 100 RU. Less is better.

7.5.15. The Chi^2 must be < 5% of R_{max}

7.5.16. The U-value must be < 15.

7.5.17. Click on the Residuals button. Ideally, the residuals will scatter randomly around zero over a range that corresponds to the short-term noise in the detection system (approximately ± 1 to 2 RU).

7.5.18. Perform a similar analysis for Test Antibody and Test Protein.

7.5.19. Select the best model that fits the data.

7.5.20. Document the association rate constant (k_a), the dissociation rate constant (k_d), the equilibrium constant (K_D), the R_{max}, and the Chi^2 results for the monoclonal antibody and protein antigen pair in a laboratory notebook.

7.5.21. Save the data to the appropriate folder in the Shared Drive.

8.0. MAINTENANCE

8.1. Daily maintenance (Excluding weekends/holidays)

8.1.1. Prepare fresh buffer if the buffer bottle volume is low.

8.1.2. Change the ultrapure deionized, 0.22 μm filtered water.

8.1.3. Check and empty the waste bottle.

8.1.4. Prime 1X to flush the system.

8.1.5. Prime 3X when switching buffers and chips.

8.1.6. Leave the instrument in *Tools: Standby* mode between experiments. Use ultrapure deionized, 0.22 μm filtered water.

8.1.7. If you intend to leave the instrument idle for more than two weeks, shut down the instrument by choosing *Tools: Shutdown*.

8.2. Weekly maintenance

8.2.1. Clean the liquid handling system by running *Desorb*.

8.3. Monthly maintenance

8.3.1. Clean the instrument cover.

8.3.2. Clean and disinfect the flow system (***Desorb and Sanitize***).Inspect the sample compartment, look for signs of flooding.

8.3.3. Check tubing connections and syringe pumps for leaks. If leaks are found, contact optirunservice.na@cytiva.com with the instrument and serial # to schedule service.

8.3.4. Run ***System Check*** if something is wrong with the instrument, i.e., lack of injections, delayed injections, or noisy data, contact optirunservice.na@cytiva.com with the instrument and serial # to schedule service.

9. REFERENCED DOCUMENTS

9.1. Biacore T200 Instrument Handbook

9.2. Biacore T200 Software Handbook

9.3. Biacore T200 Getting Started

9.4. Anti-Micro Kinetics Affinity PQ manual

9.5. Amine Coupling Kit Manual

10. APPENDIX A

10.1. Ligand Concentration Optimization for Protein A Sensor Chip

10.1.1. Check buffer volumes and empty waste container.

10.1.2. Use the same Series S Protein A sensor chip that was used in the QC.

10.1.3. Prime the instrument with 1X HBS-EP+ buffer by selecting Tools -> Prime. Follow the instructions in the dialog boxes and start the procedure. When the procedure is finished (after about 7

minutes), click on Close. A continuous standby flow of running buffer is started automatically when the procedure is finished.

10.1.4. Remove the test sample antibody. Dilute the antibody into 200 uL of 1X HBS-EP+ buffer to 1 ug/mL, 5 ug/mL, 10 ug/mL, and 20 ug/mL. Transfer 200 uL of the diluted antibodies to 4 x 0.8 mL polypropylene micro-vials and seal with a cap.

10.1.5. Add 500 uL of 10 mM Glycine pH 1.5 to a 0.8 mL polypropylene micro-vial and seal with a cap.

10.1.6. Based upon the molecular weight of the ligand antibody and analyte, determine the ideal immobilization level for a desired R_{max} of about 30 response units (RU) using the following link: surface density calculator | Cytiva (cytivalifesciences.com).

10.1.7. Open the Biacore T200 Control Software.

10.1.8. Select Run -> Manual Run to start a manual run.
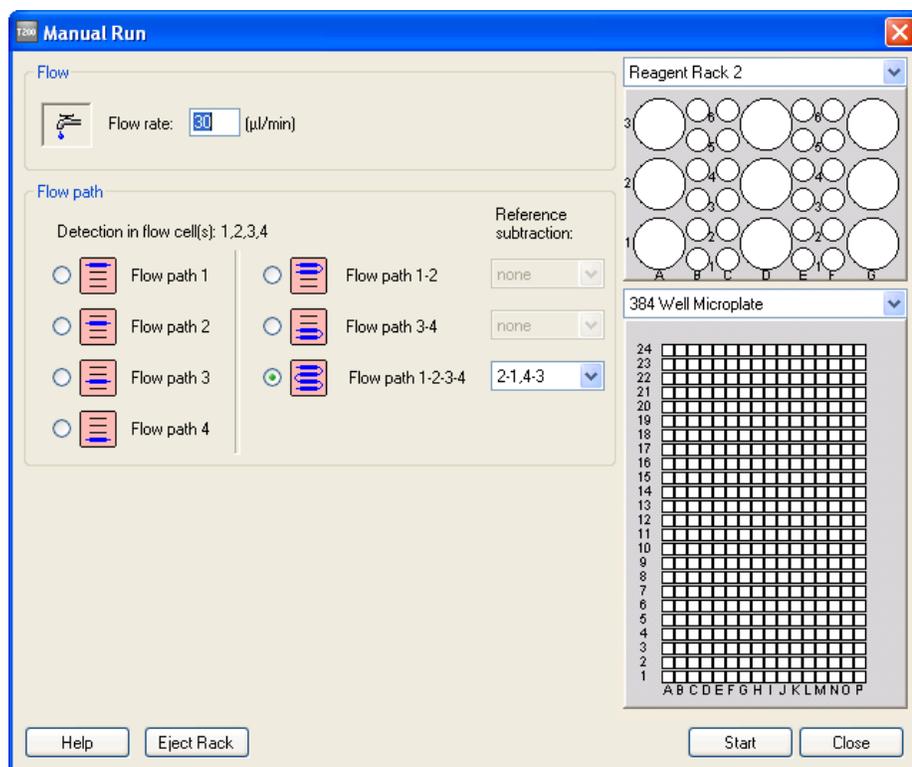
10.1.9. Select Flow path 1.

10.1.10. Click *Eject Rack* to eject the rack tray so that you can load your samples.

10.1.11. Load the samples in the appropriate positions in the rack.

Note: Document the rack positions and volumes of samples that you prepare. There is no software support in manual run for identifying samples or monitoring the volume of liquid in the autosampler position.

10.1.12. Click *Start* to start the run. Specify a result file name before the run actually starts.

10.1.13. Control the manual run from the command buttons in the main window or the options in the Command menu.

10.1.14. Use the command buttons as seen below:
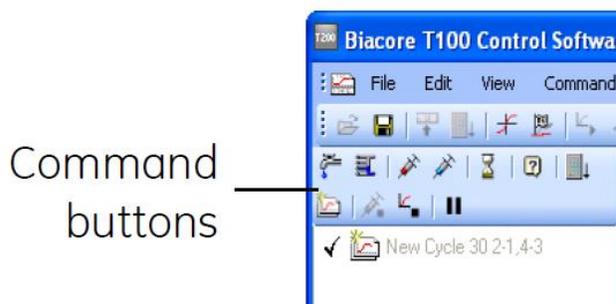
10.1.15. Select the injection command. Choose the 1 ug/mL position from which the sample will be taken and specify a contact time of 60 seconds. The required volume for the specified contact time is indicated in the dialog box. Examine the slope and the final capture level.

10.1.16. Select the regeneration command. Choose the position from which the 10 mM Glycine pH 1.5 will be taken and specify a contact time of 30 seconds. Make sure that the chosen position contains enough solution for the injection. The required volume for the specified contact time is indicated in the dialog box.

10.1.17. Repeat steps 10.1.15. and 10.1.16. for 5 ug/mL, 10 ug/mL, and 20 ug/mL.

10.1.18. Based upon the final capture level for each concentration, select the concentration that achieves an immobilization closest to the target response units (RU) calculated in step 10.1.6. If necessary, the injection time can also be adjusted to fine tune the immobilization level in order to reach the target RU.

Note: The following sections 10.2 and 10.3 are not required for optimization of ligand concentration on the Protein A sensor chip. They only apply to immobilization optimization on the CM5 chip.

10.2. Immobilization pH Scouting

10.2.1. Check buffer volumes and empty waste container.

10.2.2. Insert a new Series S CM5 chip into the machine and select CM5 in the description.

10.2.3. Prime the instrument with 1X HBS-EP+ buffer by selecting Tools -> Prime. Follow the instructions in the dialog boxes and start the procedure. When the procedure is finished (after about 7 minutes), click on Close. A continuous standby flow of running buffer is started automatically when the procedure is finished.

10.2.4. Remove the test sample antibody. Dilute the antibody to the appropriate single concentration to be tested (i.e. 5 ug/mL) into 100 uL for each of the pre-conditioning buffers 10 mM sodium acetate buffer, pH 4.0, pH 4.5, pH 5.0, and pH 5.5. Mix well. Transfer 100 uL of each pH solution into a 0.8 mL polypropylene micro-vial and seal with a cap.

10.2.5. Add 200 uL of 50 mM NaOH to a 0.8 mL polypropylene micro-vial and seal with a cap.

10.2.6. Within the Biacore T200 Control software, select *File* and *Open/New Wizard Template.* Select **Immobilization pH Scouting** under the **Surface Preparation** folder.

10.2.7. Click on new.

10.2.8. Verify the settings below and click next.

Immobilization pH Scouting - Setup

Detection
Flow path: 1

Buffers

	Buffer Name	pH
1	10 mM Acetate	5.5
2	10 mM Acetate	5
3	10 mM Acetate	4.5
4	10 mM Acetate	4
5		

Help   < Back   Next >   Close

10.2.9. Enter the appropriate name of the test antibody and concentration. Click next.

Immobilization pH Scouting - Injection Parameters

Ligand
Solution: Test Antibody
Contact time: 180 (s)   Flow rate: 5 (µl/min)

Surface regeneration
This surface wash will be run once at the end of each cycle.
Solution: 50mM NaOH

Help   < Back   Next >   Close

10.2.10. Select "Prime before Run" and click "Next".

10.2.11. The software will calculate the minimum required volumes for the run. Prepare the Reagent Rack 1 as seen below making sure to place the vials in the appropriate positions. Click next.

Position	Volume (µl)	Content	Type	Sample 1 Buffer_name
R1 A1	43	Test Antibody	Sample	10 mM Acetate 5.5
R1 A2	43	Test Antibody	Sample	10 mM Acetate 5
R1 A3	43	Test Antibody	Sample	10 mM Acetate 4.5
R1 A4	43	Test Antibody	Sample	10 mM Acetate 4
R1 B1	198	50mM NaOH	Regeneration	
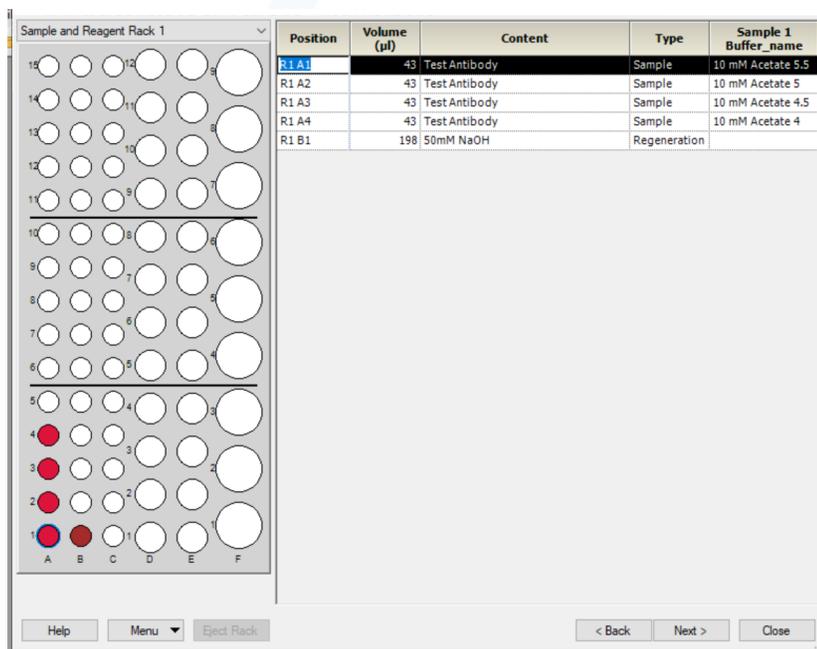
10.2.12. Verify the Run Protocol final settings and click Run.

10.2.13. **Data Analysis**

10.2.13.1. When the wizard run is completed, the results are opened automatically in the Evaluation Software, with an overlay plot of the sensorgrams adjusted to the start of the sample injection.

10.2.13.2. Choose the optimum buffer pH based upon the binding behavior: at pH suitable for immobilization, the ligand binds rapidly to the surface during the injection and dissociates completely after the end of the injection. The optimum pH is generally the highest pH value (i.e. the mildest condition) that gives sufficient ligand binding closest to the

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calculated RL, not necessarily the value that gives the highest ligand binding. Beware of conditions that give irregular sensorgrams with incomplete dissociation: this behavior often indicates aggregation or denaturation of the ligand. Use this pH to select the optimum concentration, see Appendix A, Section 10.3.

10.3. **Ligand Concentration Optimization on the Series S CM5 Sensor**

10.3.1. Check buffer volumes and empty waste container.

10.3.2. Use the same Series S CM5 chip that was used for pH scouting.

10.3.3. Remove the test sample antibody. Dilute the antibody to 5 ug/mL and 20 ug/mL each in the pre-conditioning buffer that was determined from pH Scouting (i.e. 10 mM sodium acetate buffer, pH 5.0). Mix well. Transfer 200 uL of each antibody concentration to a 0.8 mL polypropylene micro-vial and seal with a cap.

10.3.4. Determine the ideal immobilization level for a desired R_{max} of about 30 response units (RU) using the following link: surface density calculator | Cytiva (cytivalifesciences.com)

10.3.5. Within the Biacore T200 Control software, select *File* and *Open/New Wizard Template.* Select ***Immobilization pH Scouting*** under the ***Surface Preparation*** folder.

10.3.6. Click on new.

10.3.7. Verify the settings below and click next.

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Immobilization pH Scouting - Setup ✕

Detection
Flow path: 2 ⌄

Buffers

	Buffer Name	pH
1	Test Ab 5 ug/mL	5
2	Test Ab 20 ug/mL	5
3		
4		
5		

| Help | < Back | Next > | Close |
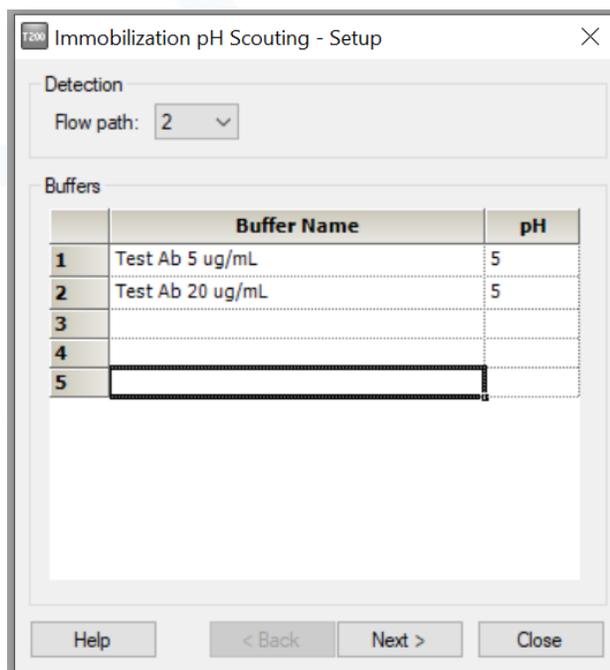
10.3.8.   Input the name of the Test Antibody. Click next.

10.3.9.   Select prime. Click next.

10.3.10. The software will calculate the minimum required volumes for the run. Prepare the Reagent Rack 1 making sure to place the vials in the appropriate positions. Click next.

10.3.11. Verify the Run Protocol final settings and click Run.

10.3.12. **Data Analysis**

10.3.12.1.   When the wizard run is completed, the results are opened automatically in the Evaluation Software, with an overlay plot of the sensorgrams adjusted to the start of the sample injection.

10.3.12.2.   Choose the optimum concentration based upon the ideal immobilization level that was calculated in section 10.3.4. to achieve an R_{max} of 30 response units (RU). Move forward with initial kinetics experiments using this concentration.