Procedure

Calibration-free concentration analysis (CFCA) in Biacore T200 using Getting Started reagents: antigen as ligand

This protocol describes how to set up a calibration-free concentration assay (CFCA) for determining the concentration of anti-β-2-microglobulin from the Getting Started Kit. The instructions apply to Biacore™ T200 software. The assay is run using a method defined in ***Method Builder***. To perform the exercise, you will need Sensor Chip CM5, Amine Coupling Kit and Getting Started Biacore T200 Kit.

Note: CFCA determinations are more robust if more than one dilution of the same sample is analyzed. This protocol describes analysis of the sample at two dilutions.

Protocol summary

The following steps are included:

- Immobilization of β-2-microglobulin
- Method definition for CFCA
- Assay: Run 2 dilutions of anti-β-2-microglobulin
- Evaluation of the results

Approximate time requirements

Preparation and immobilization: 40 min
Assay: 50 min

Ligand properties

Ligand: β-2-microglobulin
Molecular weight (M_r): 11 800
Stock concentration: 100 µg/mL

Analyte properties

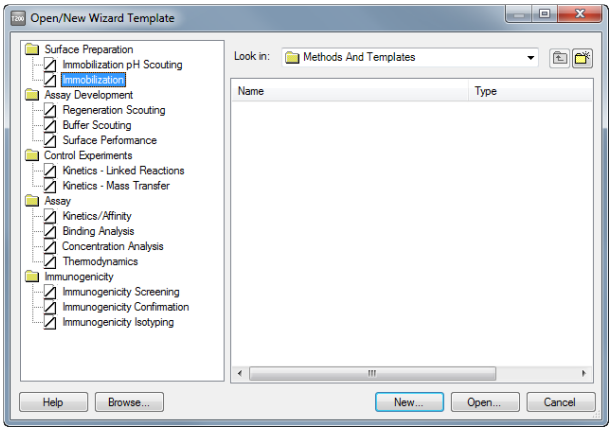
Analyte: anti-β-2-microglobulin
Molecular weight: 150 000
Stock concentration: 1 mg/mL
Diffusion coefficient at 20°C: 5.09×10^{-11} m^2/s

Ligand immobilization

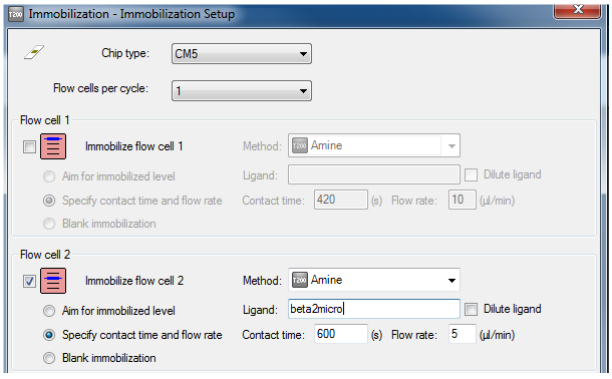
Immobilize the ligand in flow cell 2 or 4 using amine coupling.

Procedure

1. Use 10 mM sodium acetate pH 4.5 as immobilization buffer and HBS-EP+ as running buffer.
2. Dilute the ligand (β-2-microglobulin) from stock solution (100 µg/mL) to 30 µg/mL in immobilization buffer.
   - Add 30 µL ligand stock solution to 70 µL immobilization buffer.
3. Start Biacore T200 Control Software and select ***File:Open/New Wizard Template***. Choose ***Immobilization*** and click ***New***.



4. Set up immobilization of ligand in flow cell 2 or 4 on Sensor Chip CM5, using contact time 600 s and flow rate 5 µL/min). Do not use the ***Dilute ligand*** option. Leave the other flow cells blank.



5. In ***System Preparations***, check ***Prime before run***.
6. Prepare the reagent rack according to the instructions and run the immobilization.

Result

You should reach an immobilization level of about 3000 RU. The immobilization level is not critical.

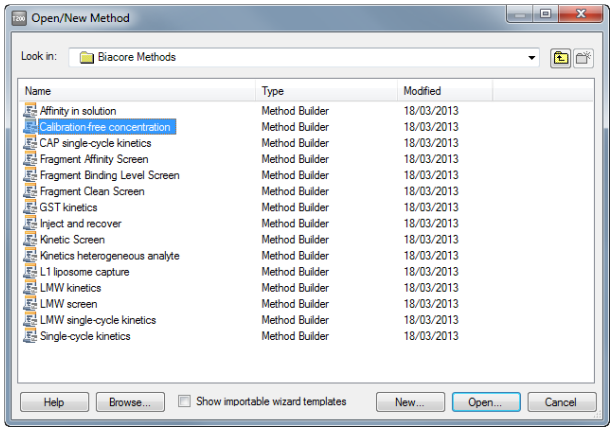
Preparing samples and reagents

1. Use HBS-EP+ as running buffer.
2. Prepare two separate dilutions of analyte (anti-β-2-microglobulin) from stock solution (1 mg/mL) to 10 µg/mL and 2 µg/mL in running buffer.
   - Add 5 µL of analyte stock solution to 495 µL of running buffer (final concentration, 10 µg/mL). Label this 100× dilution.
   - Add 80 µL of 100× dilution to 320 µL of running buffer (final concentration, 2 µg/mL). Label this 500× dilution.
3. Use Glycine 2.5 (10 mM glycine-HCl pH 2.5) as regeneration solution.

Setting up the CFCA method

Use the default Biacore method for CFCA as the starting point for your method. The description below identifies parameters that need to be adjusted. Keep the default settings unless otherwise instructed.

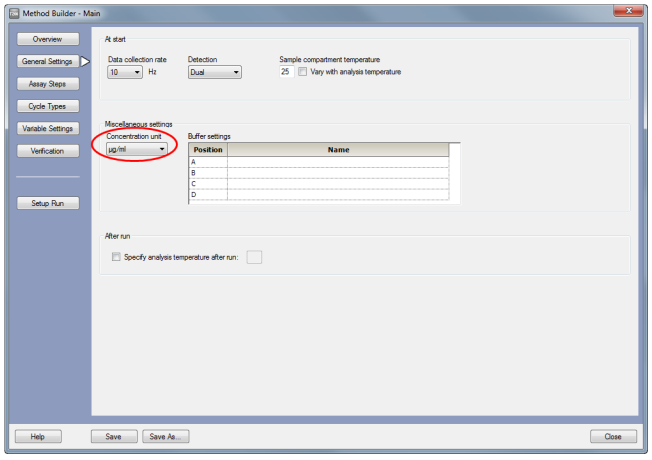
1. In Biacore T200 Control Software, choose ***File:Open/New Method***. Double-click on ***Biacore Methods*** in the dialog box and choose ***Calibration-free concentration***. Click ***Open***.



2. Modify the method settings as described below.

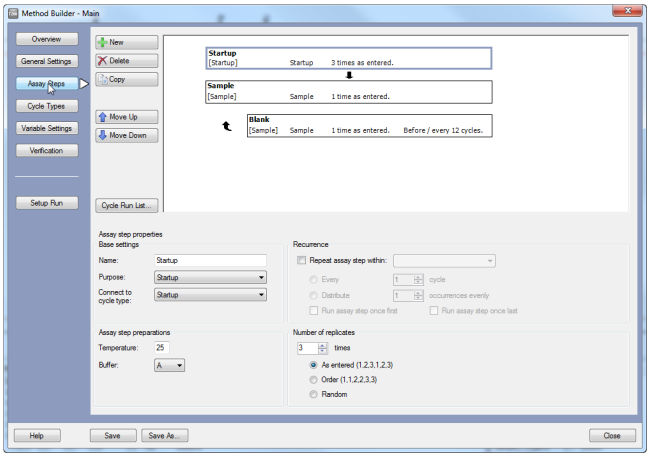
General settings

Change the ***Concentration unit*** to µg/mL.



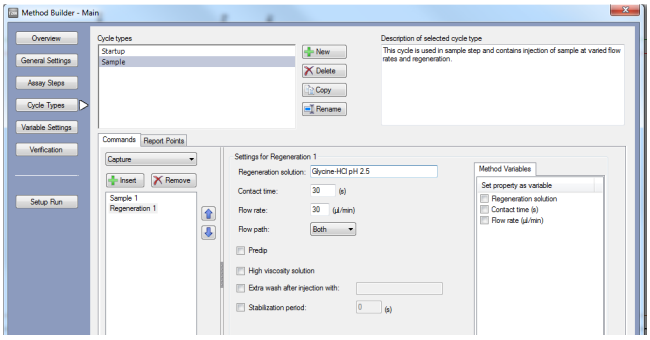
Assay steps

Leave unchanged.

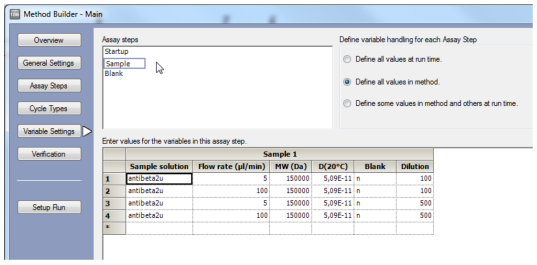
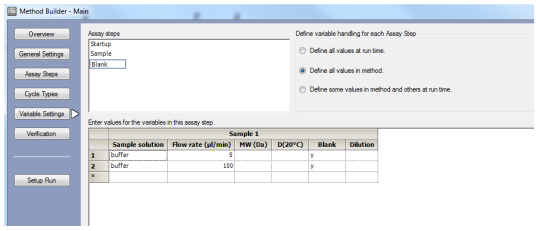


Cycle types

For both ***Startup*** and ***Sample*** cycle types, select ***Regeneration 1*** and enter ***Glycine-HCl pH 2.5*** as regeneration solution. Leave all other settings unchanged.



Variable settings

Assay step	Settings
Start-up	No variables.
Sample	Enter the values for these variables as follows: ***Sample solution*** antibeta2u ***Flow rate*** 5 and 100 ***MW*** 150000 ***D(20°C)*** 5.09e-11 ***Dilution*** 100 and 500 **Note:** You enter the value for the diffusion coefficient D at 20°C, regardless of the temperature at which the assay is run. The software adjusts the value automatically to the run temperature. Leave the value for ***Blank*** as ***N***. 
Blank	Enter the ***sample solution*** and ***flow rates***. ***Blank*** as ***Y***. 

Note: You can also define all variables at run time or in the method if you prefer.

Verification

Click ***Verification*** and check that your method is error-free.

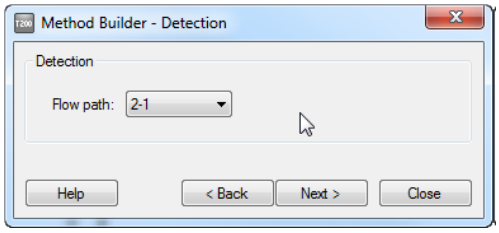
Note: This checks that the method is syntactically correct and can be used to start a run but does not check that parameter values have been entered correctly. You may save the method at this stage or continue to ***Setup Run***.

Setting up the run

1. Click ***Setup Run*** in ***Method Builder*** and adjust the settings for each step as described below.

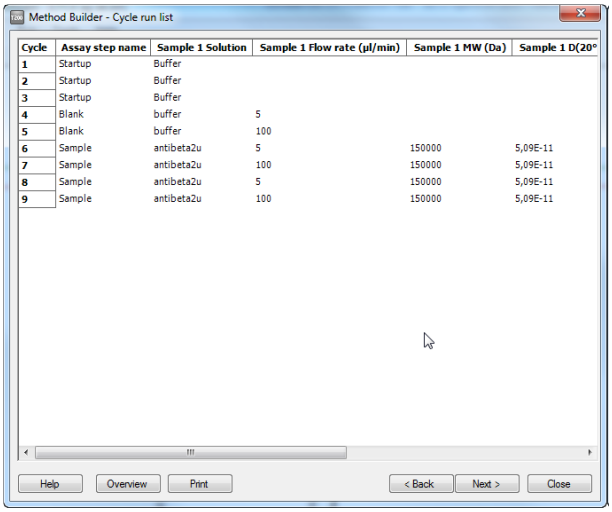
Detection

2. Choose ***Flow path 2-1*** or ***4-3***, depending on whether you have immobilized ligand in flow cell 2 or 4 respectively.



Cycle run list

3. Check that the cycle run list is correct. There should be
   - Three start-up cycles
   - Two blank cycles at flow rates of 5 and 100 µL/min
   - Four sample cycles at flow rates of 5 and 100 µL/min for dilutions 100 and 500 respectively (you will need to scroll to the right to see the dilution values).

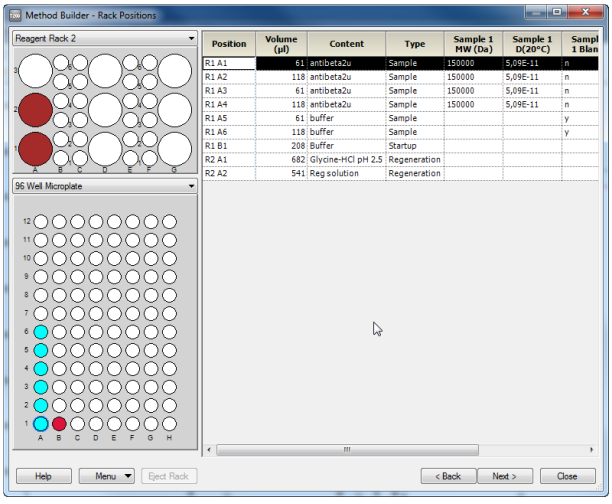


Cycle	Assay step name	Sample 1 Solution	Sample 1 Flow rate (µl/min)	Sample 1 MW (Da)	Sample 1 D(20°
1	Startup	Buffer			
2	Startup	Buffer			
3	Startup	Buffer			
4	Blank	buffer	5		
5	Blank	buffer	100		
6	Sample	antibeta2u	5	150000	5,09E-11
7	Sample	antibeta2u	100	150000	5,09E-11
8	Sample	antibeta2u	5	150000	5,09E-11
9	Sample	antibeta2u	100	150000	5,09E-11

4. In the ***System Preparations*** dialog, check ***Prime before run***.

Rack positions

5. Prepare the microplate and reagent rack as instructed in the ***Rack Positions*** dialog then start the run.



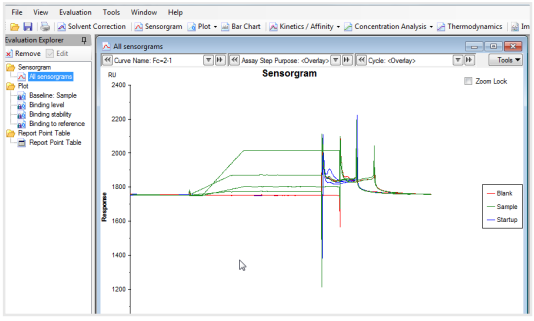
Position	Volume (µl)	Content	Type	Sample 1 MW (Da)	Sample 1 D(20°C)	Sampl 1 Blan
R1 A1	61	antibeta2u	Sample	150000	5,09E-11	n
R1 A2	118	antibeta2u	Sample	150000	5,09E-11	n
R1 A3	61	antibeta2u	Sample	150000	5,09E-11	n
R1 A4	118	antibeta2u	Sample	150000	5,09E-11	n
R1 A5	61	buffer	Sample			y
R1 A6	118	buffer	Sample			y
R1 B1	208	Buffer	Startup			
R2 A1	682	Glycine-HCl pH 2.5	Regeneration			
R2 A2	541	Reg solution	Regeneration			

The run will take about 50 min.

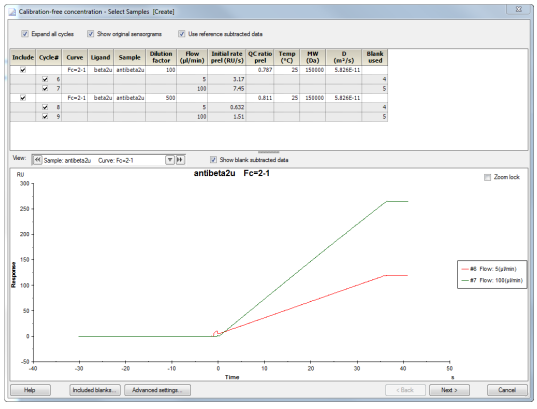
Evaluating the results

Use the ***Help*** function in the software or refer to the Biacore T200 Software Handbook for details of how to work with the evaluation software.

1. Open your result file in Biacore T200 Evaluation software.



2. Select ***Concentration Analysis:Calibration-free*** from the toolbar.

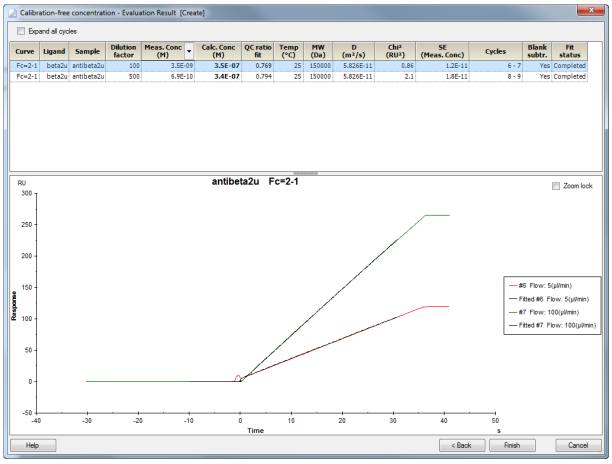


3. Check through the sensorgrams and preliminary data for general quality using the ***View*** control. Exclude any samples or cycles that are unacceptable.

Parameter	Comment
Initial rate (prel)	This is a preliminary estimate of the initial binding rate. The rate should be high enough to be reliably measurable (in practice above about 0.2 to 0.3 RU/s) and should be clearly higher at the higher flow rate.
QC ratio (prel)	The QC ratio is an indication of the extent to which binding of analyte is limited by mass transport (which is a requirement for reliable CFCA determination). A value of 1 indicates complete mass transport limitation. Lower values indicate increasing contribution of kinetic limitation. The value reported at this stage is a preliminary estimate. Exclude samples where the QC ratio is less than about 0.2.

Note: Evaluation requires at least two sensorgrams with different flow rates for each sample. If cycles are excluded so that this condition is not fulfilled, the sample concerned will not be evaluated.

4. Click ***Next*** to present the results.



5. In assessing the results, judge both the quality of fit and the reported parameters. Pay particular attention to the following:

Parameter	Comment
Meas. Conc	The measured concentration is the value determined in the actual sample after dilution.
SE (Meas. Conc)	The standard error of the measured concentration is an indication of the significance of the value. The reported concentration may not be reliable if the SE value is a significant fraction of the measured concentration.
Calc. Conc	The calculated concentration applies to the original solution before dilution. The value is the product of the measured concentration and the dilution factor. If dilutions have been performed accurately, the calculated concentration should be the same for different dilutions of the same sample.
QC ratio (fit)	This is the final value for the QC ratio, calculated from the fitted curves. Discard measurements where the QC ratio is less than about 0.2.

Note: The value shown for the diffusion coefficient D is converted from the value you entered as the temperature of the assay.

6. Click ***Finish*** to save your results or ***Back*** to review the sample selection step.

Ordering information

Product	Product code
Sensor Chip CM5, pack of 1	BR100399
Amine Coupling Kit	BR100050
Getting Started Biacore T200	28980886

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CY14996-26Oct20-PD