Calibration-free concentration analyses (CFCA) using the anti-β2-microglobulin/ β2-microglobulin model system in Biacore™ X100

This protocol describes the different steps in setting up a CFCA assay for determining the concentration of β2-microglobulin and applies to analyses in Biacore X100 (software version 2.1 or later is required).

To execute the exercise, you need the reagents in Amine coupling kit and in Biacore Getting Started Kit.

Note: *CFCA is supported in a separate wizard (located under Other Options /Wizards) and is not included as a part of the Assay Workflow. You will therefore perform both the immobilization and the CFCA assay outside the Assay Workflow.*

General recommendation for CFCA

Run **two** dilutions in **duplicate** in order to test consistency of results and to verify assay performance.

The following steps are included in the exercise:

- Immobilization of anti-β2-microglobulin using immobilization wizard.
- Set up a new CFCA wizard template.
- CFCA assay: Run 2 dilutions in duplicate of β2-microglobulin.
- Evaluate the results.

Time estimate

Immobilization: ~40 min (incl. prime and normalize)
CFCA assay: ~2 h (excl. prime)

Ligand

Molecular weight of ligand (anti-β2µ-globulin): 150 kDa
Stock concentration: 1 mg/mL

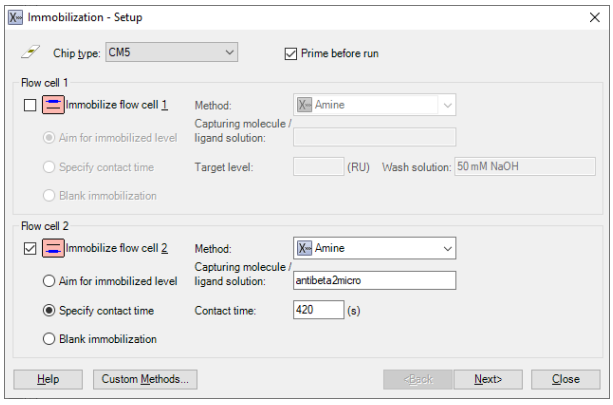
Analyte

Molecular weight of analyte (β2µ-globulin): 11.8 kDa
Stock concentration: 100 µg/mL
Diffusion coefficient: 1.19 × 10^{-10} m^2/s (at 20°C)

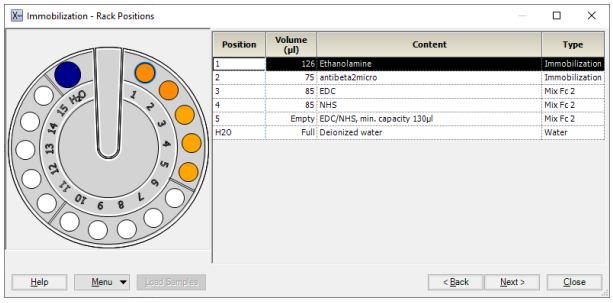
Immobilization

1. In this exercise you will use the Immobilization wizard provided with the control software. Click on **Other Options**. Click on **Wizards** and choose **Immobilization**. Click **New**.

2. Choose **Chip type**: CM5 and **Immobilize flow cell 2** and **Method**: Amine. Enter **Ligand solution**: antibeta2micro. Choose option **Specify contact time** and use **Contact time**: 420 s. Click **Next**.



3. Prepare your samples: Dilute the ligand (monoclonal anti-β2µ-globulin) in immobilization buffer to 30 µg/mL (concentration in stock solution: 1 mg/mL). Add 6 µL ligand to 194 µL immobilization buffer (10 mM sodium acetate pH 5.0). Fill vials with Ethanolamine, EDC, NHS and deionized water according to the software. Remember to add an empty vial in the rack (for mixing of EDC and NHS). Use HBS-EP+ as running buffer.



4. Click on **Load samples** to eject the sample and reagent rack. Place the vials according to the **Rack Positions** table.

5. Place the sample and reagent rack in the correct position in the sample compartment and click **OK** in the **Insert Rack Tray** dialog.

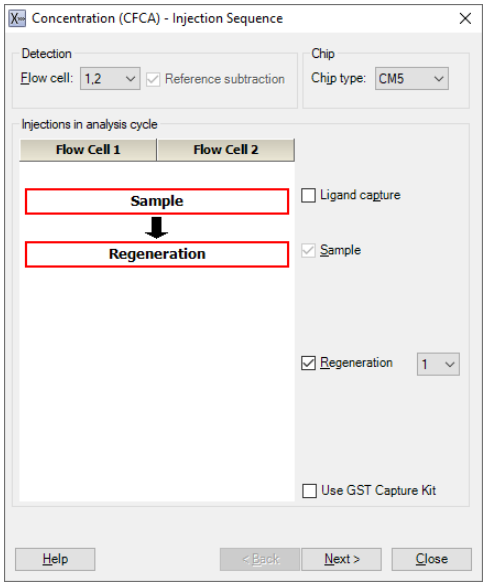
6. Click **Next** in the **Rack Positions** dialog.

7. Click **Start** to begin the Immobilization. Enter a name for the results and click **Save**. The immobilization run will take approximately 40 minutes. *An immobilization level around 4000-15 000 RU should be reached for this specific assay.*

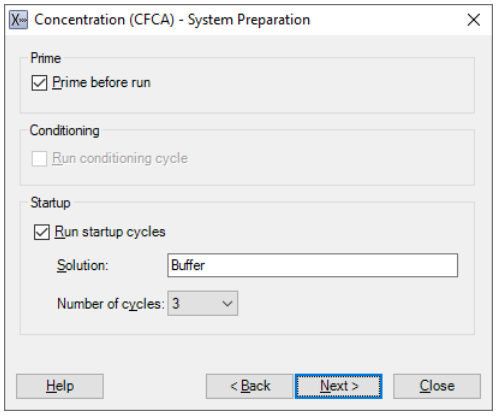
CFCA assay set-up

1. In this exercise you will use the CFCA wizard provided with the software. Click on **Wizards (Other Options)** and choose **Calibration-free (CFCA)**. Click **New**.

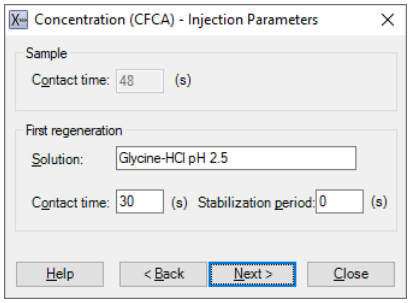
2. Use the default settings in the Injection sequence step. Click **Next**.



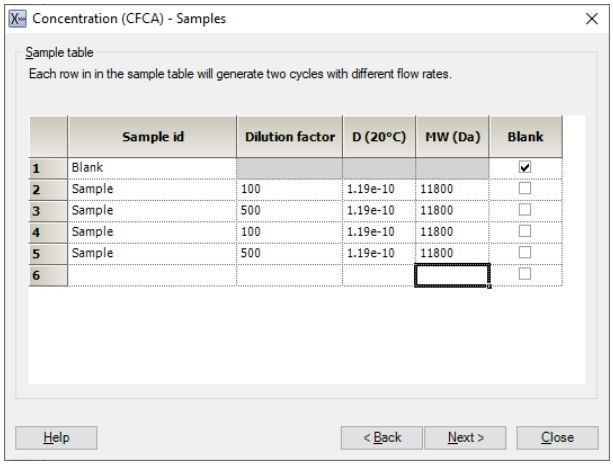
3. Use the default settings in the System preparation step. Enter **Solution**: Buffer (for startup cycles). Click **Next**.



4. The **Contact time** for the sample is fixed to 48s. Enter the regeneration **solution**: Glycine-HCl pH 2.5 and use the default settings for contact time and stabilization period. Click **Next**.



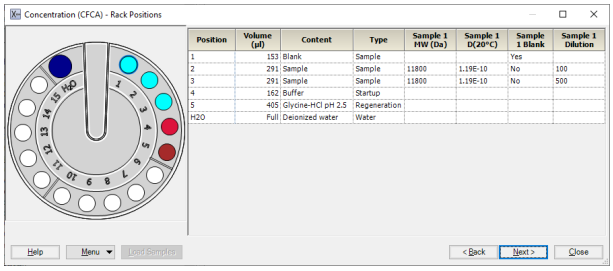
5. Enter **Sample id**, **Dilution factor, Diffusion coefficient (D = 1.19 × 10^{-10})** and **MW (11800 Da).** In this exercise you will run 2 concentrations: 1 µg/mL (diluted 100 times) and 0.2 µg/mL (diluted 500 times). Click **Next**.



6. Prepare your samples:
   a) **Dilution 100x.** Dilute the β2µ-globulin to 1 µg/mL (concentration in stock solution: 100 µg/mL) in running buffer. Add 5 µL analyte to 495 µL running buffer.
   b) **Dilution 500x.** Dilute the β2µ-globulin to 0.2 µg/mL. Add 100 µL of the 1 ug/mL dilution to 400 µL running buffer.

7. Fill vials with samples, running buffer (both for blank and startup), regeneration solution deionized water according to the software. Use HBS-EP+ as running buffer.

8. Click on **Load samples** to eject the sample and reagent rack. Place the vials according to the Rack Positions table.



9. Place the sample and reagent rack in the correct position in the sample compartment and click **OK** in the Insert rack dialog.

10. Click **Next** in the Rack positions dialog.

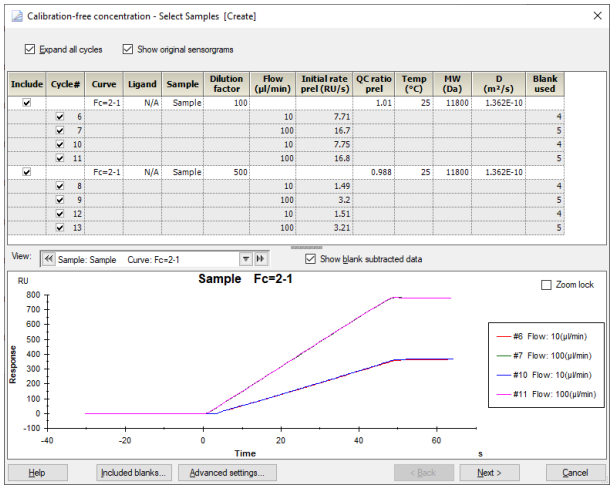
11. Click on **Start** to begin the CFCA assay. Enter a name for the results and click Save. The run will take approximately 2 hours.

Evaluation - Interpreting the results

The data from your CFCA assay is automatically opened in the evaluation software. Use the predefined evaluation tool for CFCA.

1. Click on **Concentration Analysis** and then choose **Calibration-free**.

2. Selecting samples
   a) Click **Included blanks** to display an overlay plot of blank cycles in the run. Remove the checkmarks from the "Include" column to remove a blank cycle from the evaluation if required. Click **OK** to get back to the Select samples step.
   b) Examine the sensorgrams in the lower panel. Sensorgrams at the lower flow rate should be approximately linear throughout the sample injection (with allowance for short disturbances at the beginning and end of the injection). Exclude cycles with obviously disturbed sensorgrams, or with markedly non-linear sensorgrams at the lower flow rate.



   c) Examine the values in the **QC ratio** column. The QC ratio is an indicator of the extent to which the initial binding rate is influenced by flow rate. Exclude samples with a QC ratio of less than about 0.2.
   d) Examine the initial rate. Results from samples where the initial binding rate at 10 µL/min is below 0.3 should be treated with caution. As a general rule, the upper limit of the initial binding rate at 10 µL/min is analyte MW in kDa/10. For example, for an analyte of molecular weight 150 kDa the acceptable range is about 0.3–15 RU/s. However, there is no sharp boundary here. If the sensorgram is linear during injection (despite higher initial binding rate), it can often be used for the analysis.

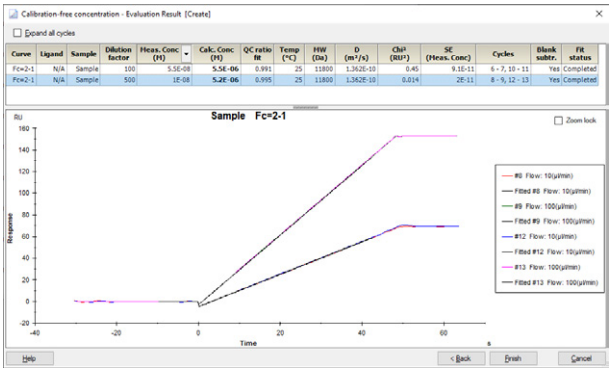
      Remember that each sample must be represented by at least two cycles with different flow rates to enable evaluation.

3. Selecting data

   If required, remove disturbed data from one or more sensorgrams. Click **Advanced** and drag with right mouse button to select data and click **Remove selection**. You can work with either all or single sample series (Settings panel). You can also adjust the fitting range (left and right) with the vertical blue lines. Click **Close** to get back to the Select Samples step. Click **Next** to continue with the evaluation.

   Note: *Evaluation is performed using data from 10 s before injection start to 5 s before injection stop. You do not need to remove disturbances if they are outside these limits.*

4. Evaluating results
   a) Examine the fitted curves in relation to the experimental data. Discard samples where the fit is poor at one or both flow rates. As a general rule, the value reported for chi-squared should be less than about 5% of the maximum response reached at the lowest flow rate (disregarding the difference in units between chi-squared and response).
   b) Examine the statistical significance of the reported concentration as indicated by the standard error or T-value. The standard error should be 10% or less of the reported concentration. The T-value should be greater than 10. Discard samples where the standard error represents more than 20% of the reported concentration or the T-value is less than 5. Treat results where the standard error is in the range 15%–20% of the concentration (T-value 5–10) with caution.
   c) Check the QC ratio. Reject samples with a QC ratio of less than about 0.2.
   d) Examine the reported concentration values. The optimal concentration range that can be measured with calibration-free concentration analysis is 0.5–50 nM. Values outside this range should be treated with caution.
   e) Click **Finish**.



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