Chapter 16

Measuring Poliovirus Antigenicity by Surface Plasmon Resonance. Application for Potency Indicating Assays

Janny Westdijk, Larissa van der Maas, Rimko ten Have, and Gideon Kersten

Abstract

The D-antigen ELISA is the commonly accepted test for release of inactivated poliovirus containing vaccines. However, this test has a few drawbacks regarding the many variations in the method to quantify the D-unit. The result may depend on method and reagents used which makes standardization of inactivated polio vaccines, based on D-units, to a real challenge. This chapter describes a surface plasmon resonance based method to quantify D-units. The advantage of the calibrated D-antigen assay is the decrease in test variations because no labels, [no incubation times] and no washing steps are necessary. For standardization of both IPV and Sabin IPV, the calibration free concentration analysis could be an improvement as compared to ELISA or other SPR methods because this method combines quantity (particle concentration) and quality (antigenicity) in one assay.

Key words D-antigen ELISA, Surface plasmon resonance, Calibrated D-ag assay, Calibration free concentration assay, Polio vaccine, Sabin, IPV

1 Introduction

For release and stability studies the active component in polio vaccines is measured by a sandwich ELISA in which D-ag specific antibodies are used to determine the D-ag concentration [1]. Also the rat potency test [2, 3] is used. Since 2005 the European Pharmacopoeia allows waiving of the rat potency and to rely exclusively on the ELISA as a potency indicating test for release of inactivated poliovirus containing vaccines [4].

However, the ELISA has a few drawbacks. The most important is that there is no common inter-laboratory method to quantify the D-antigen unit [5]. The methods differ in the type of (catching and detecting) antibodies. Regarding the antibodies, which could be polyclonal or monoclonal, infinite combinations of detecting and catching antibodies are possible. If the detecting antibody is labeled, even more alternatives are possible. The method can also

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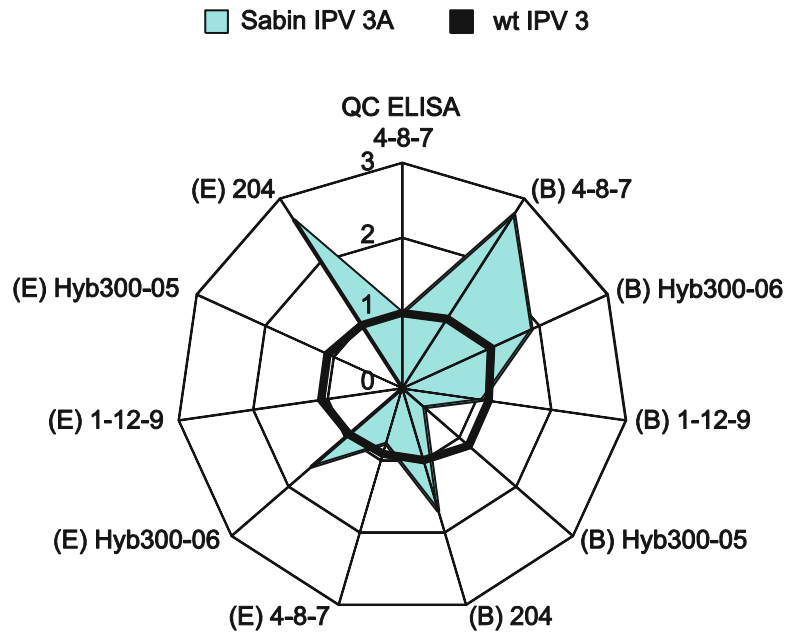

Fig. 1 Antigenic fingerprint of Sabin and wt IPV Type 3. The ***right half*** of the radarplot represents the ratio D-antigen concentration obtained by Biacore (marked (B)) to the D-antigen concentration obtained by QC-ELISA with mab 4-8-7. At the ***left half*** of the radarplot D-antigen ratio's by ELISA are shown (marked (E)). Reproduced from Vaccine, 2011 [6] with permission from Elsevier

differ in buffers used, incubation times, the sort of conjugation, blocking steps, and washing steps. For instance, differences in incubation time and temperature (2 h at 37 °C versus 2 h at 37 °C and overnight at 4 °C) resulted in a twofold difference in the apparent D antigen concentration [6]. Furthermore, many different monoclonal antibodies (mabs) are available for each serotype. These mabs could also be the reason for a variation in D-antigen concentration. With regard to inactivated poliomyelitis vaccine (IPV) this variation is not that pronounced; however, the D-antigen concentration of Sabin IPV (sIPV) depends strongly on both method and mabs, when using anti-Salk mabs (Fig. 1) Because of the many variations in the method to quantify the D-antigen unit, which may result in considerable inter-laboratory variability [7], standardization of IPV remains under discussion. If, in the foreseeable future, Salk IPV will be replaced by sIPV, which could be a serious option [8], standardization of sIPV will even be more of a challenge, considering the dependence of the D-unit on method and mabs.

Surface plasmon resonance (SPR) technology does not solve the sIPV dependency on mabs which is also illustrated in Fig. 1. SPR calibrated concentration assays use the same format as ELISA's for the quantification of antigens: first an anti-mouse antibody is

immobilized to the surface of a chip, then a complex is formed by subsequent injections of an antigen specific mouse mab and a captured antigen. However, the advantages of SPR-measurements are that the measurements are label-free where the ELISA requires a conjugated antibody. The ELISA requires also several washing steps, Biacore detects the vaccine directly. Furthermore, the detection is in real-time: the Biacore monitors continuously each binding step in the assay, allowing visual monitoring each step, where the result of the ELISA is only seen after the final step. Last but not least, the hands-on time of an ELISA is quite longer than that of a Biacore assay. Besides, the inter-laboratory variability might decrease substantially because of the lacking of incubation steps, intermediate washing steps and different sets of polyclonal/monoclonal antibodies. Finally, results from Biacore correlate well with D-units from ELISA (Fig. 2). Also the lower limits of quantification are more or less the same: 3–0.4–1.1 DU/ml (type 1, 2, and 3, respectively) for Biacore and 1.5–0.4–0.7 DU/ml for ELISA.

Biacore measurements rely on SPR. The underlying complex theory of SPR is accurately described in the Biacore concentration analysis handbook [9]. It suffices to know that SPR-based instruments use an optical method to measure the refractive index near (within ~300 nm) a sensor surface. In the Biacore this surface forms one side of a small flow cell, through which a solution (the running buffer) passes under continuous flow. In order to detect an interaction one molecule (the ligand) is immobilized onto the sensor surface. Its binding partner (the analyte) is injected in buffer through the flow cell, also under continuous

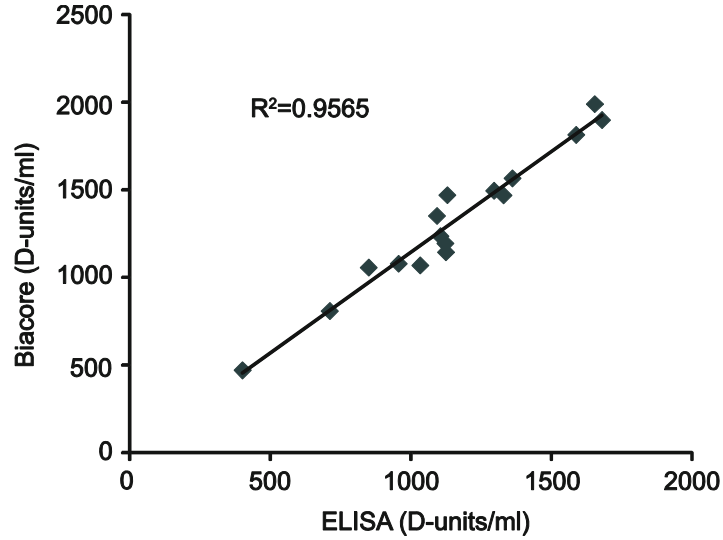

Fig. 2 Correlation between D-antigen ELISA and the calibrated Biacore D-antigen assay of 16 monovalent bulk vaccines of either type 1, 2, or 3

flow. As the analyte binds to the ligand, the accumulation of protein on the surface results in an increase in the refractive index. This change in refractive index is measured in real time, and the result plotted as response or resonance units (RUs) versus time (a so-called sensorgram) [10].

For the calibrated D-antigen assay it comes down to immobilization of anti-mouse IgG Fc-specific antibodies on the dextran layer of a sensorchip by primary amine coupling. Next, a detecting antibody is injected p, followed by IPV. The sensor chip is regenerated with 10 mM glycine-HCl, pH 1.5, preparing the surface for the next run, varying the sample or reference dilution. Assay data are analyzed by a four-parameter logistic curve fitting using the Biacore evaluation software. Antigenicity is calculated relative to the international IPV reference. This SPR method is robust and considerably more accurate as compared to ELISA. CV values below 5 % are easily achieved by this classical SPR method.

A few years ago, Biacore introduced a second approach, the calibration-free concentration analysis (CFCA). CFCA allows the determination of the absolute antigen concentration without the use of a reference. The analysis relies on measurement of the binding rate during sample injection under partially or complete mass transport limited conditions. Mass transport is a diffusion phenomenon that describes the movement of molecules from a higher concentration (e.g., in the bulk fluid in the center of a capillary) to a lower concentration (e.g., in the stagnant layer of fluid near the surface of a capillary). Mass limitations arise when either analyte binds to the surface faster than it diffuses from the solution during injection, or analyte does not diffuse fast enough from the surface during dissociation, leading to rebinding. To calculate the analyte concentration in the bulk solution a relation between initial binding rate and analyte concentration in the bulk is used. On a sensor surface with a high immobilization level the initial binding rate (slope) can be described as a function of the molecular weight of the analyte, the mass transport coefficient and the concentration of the analyte. The mass transport coefficient is calculated using flow cell dimensions, flow rate, and diffusion coefficient. The sample is run over the surface at two flow rates (5 and 100 µl/min), the initial binding rate (dR/dt) is measured and the software resolves the concentrations based on the aforementioned parameters. As long as the affinity of antigen and antibody is high enough ($k_a < 5 \times 10^4/M^{-1}\ s^{-1}$ and/or $K_D > 10^{-6}$ M), the calculated concentration is independent of the antibody used. The dynamic range of the method is approximately 0.05–5 µg/ml [9].

Indeed, results of CFCA with sIPV show that the active concentration measurement of type 1, 2 and 3 IPV (wt IPV and sIPV) is independent of the mab (*see* Fig. 3). The active particle concentration of polio vaccines also correlate with the virus concentrations of the vaccines calculated from the absorbance at 260 nm [6].

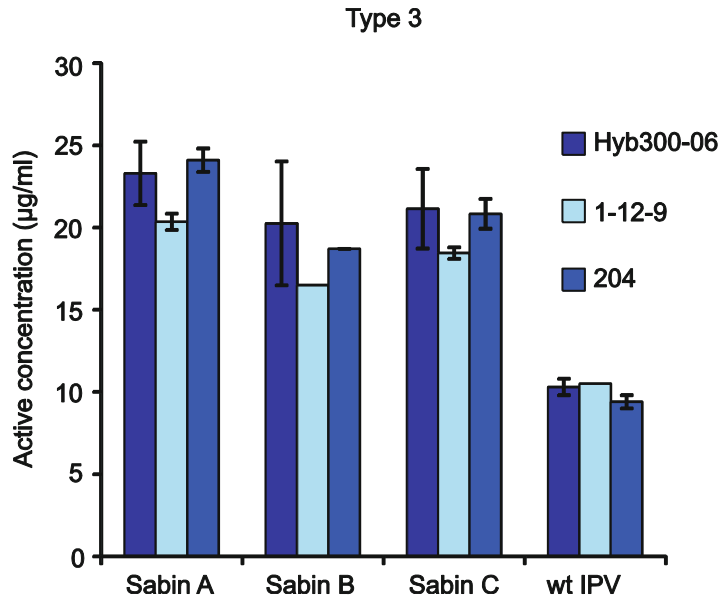

Fig. 3 Results of calibration free concentration analysis of Sabin/wt IPV type 3, resp. 15/50 DU/ml; D-units based on QC-ELISA. Error bars represent SD at $n=3$; where error bars are missing, the concentration is an average of a duplicate measurement. Reproduced from Vaccine, 2011 [6] with permission from Elsevier

Because the D-antigen unit is not well defined, IPV quantification by using protein or virus concentrations is attractive from a standardization point of view. However, use of a protein assay is only valid if almost 100 % of the protein is poliovirus and has the native conformation, i.e., D-antigenicity. Measurement of the active particle concentration by CFCA is an attractive alternative as it combines quantity and quality in one assay. CFCA requires no calibration curve and the measurement is highly specific. Differences between different mabs do not exist; all high affinity mabs give the same results. Implementation of this assay as an in vitro measurement of IPV potency would require additional validation and an international harmonization study, but it may be worth doing considering the increasing role of IPV in the world.

2 Materials[1]

1. CM3-chip (GE Healthcare) (*see* **Note 1**). Storage 4–8 °C.
2. Amine coupling kit (GE Healthcare): *N*-hydroxysuccinimide (NHS), 115 mg; 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), 750 mg; 1 M ethanolamine

[1] Identification of particular products is provided as a guide to aid in the selection of equivalent, suitable products.

hydrochloride-NaOH pH 8.5. Dissolve the EDC and NHS by adding 10.0 ml of filtered, deionized water (Milli-Q) to each vial. Cap vials tightly and agitate until the solids are completely dissolved. Dispense the EDC and NHS solutions separately in 100 µl aliquots for storage at −18 °C or below. For the aliquots use the plastic vials Ø 0.7 mm and cap the vials with the type 3 rubber caps. Use aliquots within 2 months (*see* **Note 2**).

3. Acetate 5.0 (GE Healthcare): 10 mM sodium acetate, pH 5.0. Storage 4–8 °C.
4. HBS-P buffer 10x (GE Healthcare): 0.1 M HEPES; 1.5 M NaCl; 0.5 % v/v surfactant P20 will yield pH 7.4 when diluted: supplement 900 ml of Milli-Q with 100 ml 10× HBS-P buffer 10×. Store at room temperature. This buffer will be used as running buffer and dilution buffer. Filter the diluted buffer through a 0.2 µm filter before running a method.
5. Glycine 1.5 (GE Healthcare): 10 mM glycine-HCl pH 1.5. Storage 4–8 °C.
6. Goat anti-mouse (GaM) IgG F_c fragment specific (Thermo Scientific): antibody concentration 2.3 mg/ml in 0.01 M sodium phosphate and 0.25 M NaCl, pH 7.6. Store at 4 °C. Expiration date 1 year from date of product receipt. Prepare a ten times dilution (200 µg/ml GaM IgG Fc) in 100 µl acetate 5.0 buffer in a Ø 7 mm plastic vial (*see* also Subheading 3.1 **step 11**).
7. Reference preparation poliomyelitis vaccine inactivated trivalent (Pu91-01, RIVM): 430-95-285 DU/ml for type 1, type 2, and type 3, respectively. Storage ≤−70 °C. Calibration curves of trivalent Pu91-01 are twofold dilution series of 8 dilutions, starting with 1:1 in HBS-P buffer (*see* also Table 1). Prepare fresh before use.
8. IPV serotype 1, 2, and 3 specific mouse monoclonal antibodies (mabs) Hyb295-17 (IgG2b/k); Hyb 294-06 (site 1 specific, IgG2a/k); and Hyb300-06 (site 1 specific, IgG2a/k), respectively (BioPorto Diagnostics A/S): each mab 1 mg/ml ± 15 % in 0.01 M Phosphate Buffered Saline (PBS), pH 7.0; 0.5 M NaCl and 15 mM Sodium Azide. Storage 4–8 °C (*see* **Note 3**). For the calibrated D-antigen assay, dilute each mab to 1 µg/ml in HBS-P buffer. For the calibration-free assay the mabs are diluted to 4 µg/ml in HBS-P. Prepare fresh before use.
9. Inactivated polio vaccine samples for testing (*see* **Note 4**). The vaccines must be stored at 4–8 °C. The polio vaccines are stable for 24 months at 4–8 °C. Prepare appropriate dilutions (within the range of the calibration curves) fresh before use.
10. Microplates 96-well (GE Healthcare): polystyrene microplates.

Table 1
Typical D-antigen concentrations used for calibration curves of type 1, 2, and 3 of the international reference Pu91-01[a]

	D-antigen concentration (DU/ml)		
Standard nr.	Type 1	Type 2	Type 3
1	1.7	0.4	1.1
2	3.3	0.7	2.2
3	6.7	1.5	4.5
4	13.4	3.0	8.9
5	26.9	5.9	17.8
6	53.8	11.9	35.6
7	107.5	23.8	71.3
8	215.0	47.5	142.5

[a]Twofold dilution series of Pu91-01 with starting dilution 1:1 in HBS-P buffer

11. Microplate foils (96-well) (GE Healthcare): self-adhesive, transparent plastic foils.
12. Glass Vials, Ø 16 mm (GE Healthcare): 4.0 ml borosilicate screw top glass vials
13. Rubber caps, type 2 and 3 (GE Healthcare): penetrable cap made of Kraton G. Ventilated.
14. Plastic vials, Ø 7 mm (GE Healthcare): 0.8 ml rounded polypropylene microvials (*see* **Note 5**).
15. Plastic vials, Ø 11 mm (GE Healthcare): 1.5 ml polypropylene vials with wide opening that allows a pipette to reach the bottom.
16. SFCA Serum Filter Unit (Thermo Scientific): 500 ml bottle, 0.2 μm pore size

3 Methods

Although the calibrated D-antigen assay can be performed on all available Biacore instruments, whereas the CFCA method runs at the X100 and T200 Biacore, the procedures below are based on working on a T200-instrument (Biacore control software version 2.0) with T200 evaluation software (version 1.0).

3.1 Immobilization

Before performing the calibrated D-antigen assay or the calibration free assay, the sensor chip must be prepared enabling to capture the anti-polio mouse monoclonal antibodies. For this purpose

Goat-anti mouse IgG Fc specific antibodies are covalently bound to a CM3 sensorchip. In case of performing a calibration-free concentration assay, a second flow cell must be prepared with a blank immobilization.

1. Open the Biacore T200 control software (*see* **Note 6**).
2. Click on **insert sensor chip** icon. A window appears: **this will eject the sensor chip**.
3. Click on **Eject Chip** (this will take a minute).
4. A dialog box **Insert Chip** appears. Click on where appropriate, either **new chip** or **reuse**. In case of a new chip, select the chip type **CM3**, enter a chip id and chip lot number. In case of a reused chip, select the chip id from the pull-down menu. Close the sensor chip port cover and click **Dock Chip** to dock the chip in the instrument.
5. Click **File**, then **Open/new wizard template**, and then click **Immobilization** from the **Surface preparation** directory.
6. Click on **New** or **Open** an existing immobilization file.
7. The dialog box **Immoblization setup** appears (Fig. 4). Select **CM3** as **Chip type**. Select flow cell **1, 2, 3, or 4** (calibrated D-antigen assay)or in case of CFCA flow cells **1** or **3 blank** and **2** or **4 anti-mouse IgG Fc**. Select as **Method**: **Amine (CFCA)** or **Amine + Regeneration 3× (calibrated D-antigen assay)** (*see* **Note 7**). Enter the **ligand** Goat anti Mouse IgG Fc (for **CFCA** in either flow cell 2 or 4). Tick off **Specify contact time and flow rate. Contact time** 420 (s), **flow rate** 10 (µl/min). Click in case a CFCA is performed, in either flow cell 1 or 3 **blank immobilization**. Press **Next**.
8. The window **System preparations** appears. Tick off **Prime before run**. Enter **analysis temperature**, 25 °C, **compartment temperature** 25 °C. Press **Next**.
9. The window **Rack positions** appears. This dialog box shows where samples and reagents are placed in **reagent** rack 2 or **sample and reagent** rack 1 (*see* **Note 8**). Positions are color-coded according to sample and reagent categories. Positions are described by tool tips (hold the cursor over the position for a couple of seconds to display the tool tip). Empty positions show the position capacity and dead volume. Used positions show in addition the content name and the volume that will be used.
10. If the rack is already placed in the compartment, click in the dialog box **Eject rack**. The rack tray is ejected.
11. Place all necessary samples in the required volumes and concentrations (*see* **Note 9**) in the appropriate positions

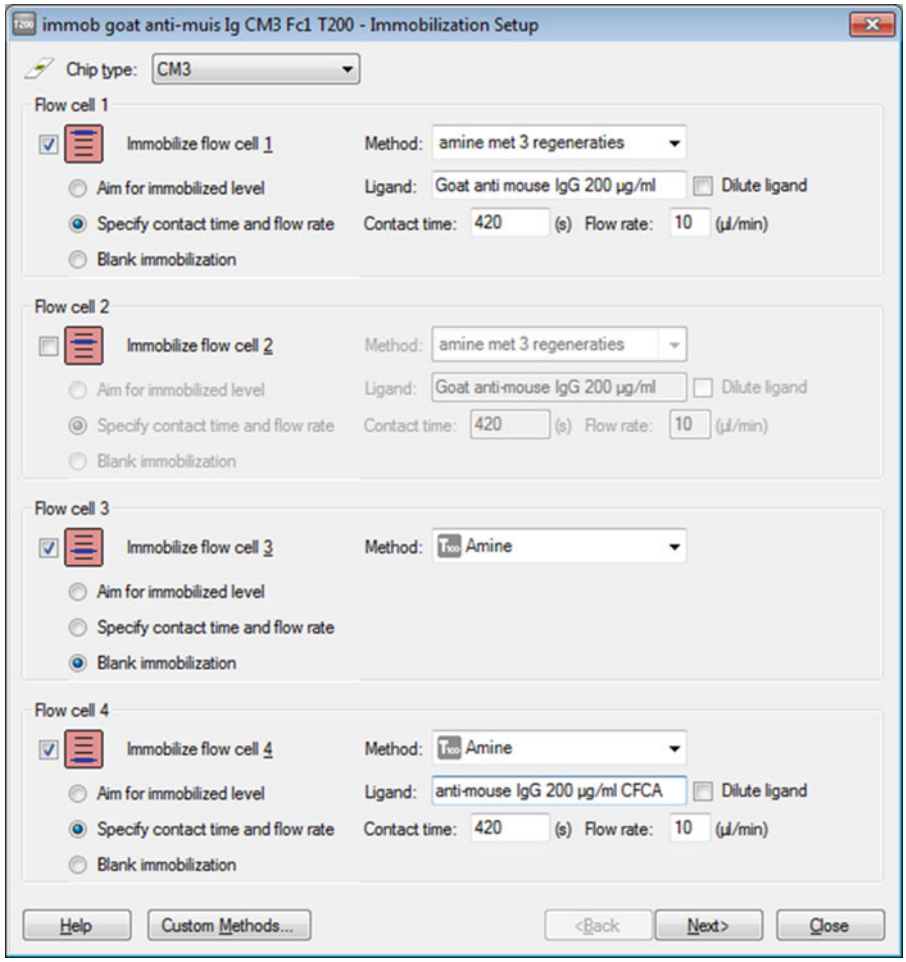

Fig. 4 Example of immobilization setup screen

according to the dialog box: frozen aliquots of EDC and NHS, 130 µl Ethanolamine, Goat-anti mouse IgG (*see* also heading 2 **item 6**) in acetate pH 5 buffer, and glycine-HCl (Fig. 5).

12. Click again **Eject rack**. The rack compartment opens. Place the rack into the compartment and click **OK**. Click **Next**.
13. A window **Prepare Run Protocol** appears. Perform the instructions in the dialog box (*see* **Note 10**). Click on **Save as** and enter a name for the immobilization method. Click on **Run** and enter a file name for the immobilization results.
14. After the immobilization run is finished (Fig. 6), a window **Immobilization Results** appears. This dialog box summarizes the results of the immobilization (*see* **Note 11**).

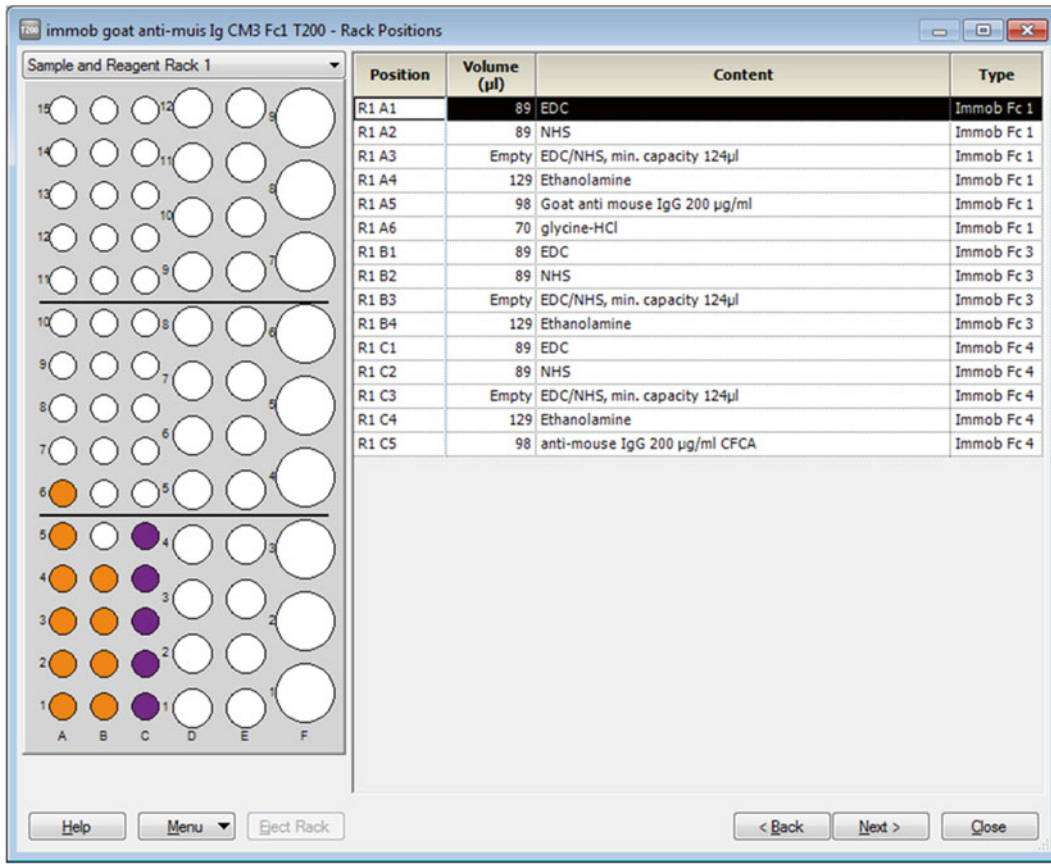

Fig. 5 Example of Rack positions screen. Immobilization set up for Row A: amine + 3 regenerations method; row B: blank immobilization; row C: amine method

3.2 Calibrated D-Antigen Assay for One Serotype and One mab

If samples consist of just one serotype, e.g., monovalent bulk, the concentration assay can be performed following the T200 concentration wizard. If samples are trivalent or it is feasible (considering the number of samples, *see* **Note 12**) to measure three serotypes in a row, it is better to build up a method (*see* Subheading 3.3).

1. Open the Biacore T200 control software.
2. Click **File**, then **Open/new wizard template**, and then click **Concentration Analysis** from the **Assay** directory.
3. Click on **New** or **Open** an existing concentration analysis file.
4. The window **Injection Sequence** appears. Choose the flow path (refers to the flow cell that has been immobilized) for the experiment. Choose the chip type (CM3) for the experiment. Select the sequence of injections for the assay: **Ligand capture** (refers to the use of anti-polio serotype specific mab), **Sample** (refers to the IPV sample injection), **Regeneration** (refers to the injection of regeneration solution to remove bound

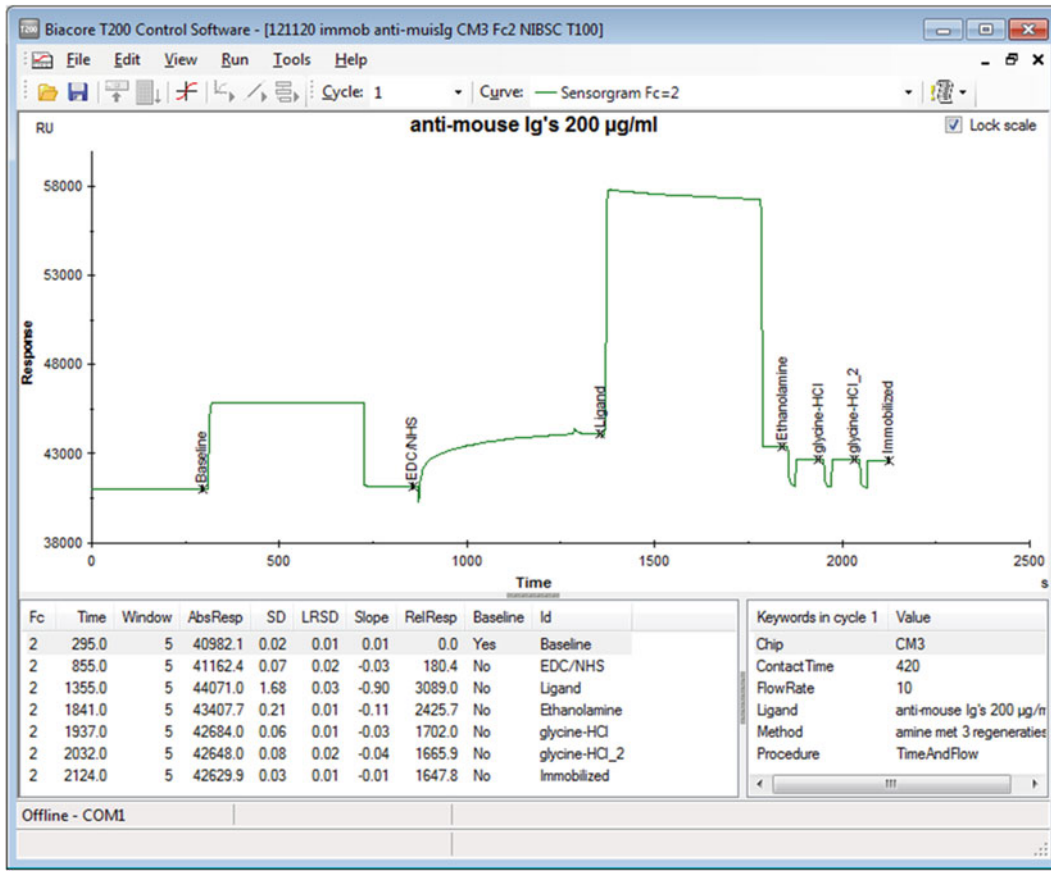

Fig. 6 Sensorgram immobilization Goat anti-Mouse IgG Fc by amine coupling with three regenerations

analyte) (the complex of mab and vaccine) from the surface. Click 1 injection for the regeneration. Click **Next**.

5. The window **Injection Sequence** appears. Click **Run startup cycles** to include dummy analysis cycles at the start of the run to make sure the system is stable. Enter HBS-P for the solution. Enter 5 for the number of cycles. Click **Next**.
6. The window **Injection Parameters** appears. Enter successively: **ligand name** the name of the serotype specific mab, **Contact time** 120 (s), **flow rate** 10 (µl/min), **Stabilization period** 0 (s), **Sample Contact time** 120 (s), **flow rate** 10 (µl/min), **Regeneration solution** Glycine-HCl 1.5, **Contact time** 30 (s), **flow rate** 30 (µl/min), **Stabilization period** 0 (s). Click **Next**.
7. The window **Calibration Parameters** appears. Enter the name of the International Reference used for the calibration curve. The option **Run First** is always checked, and indicates that a calibration curve will always be run before the first sample or control sample cycle. Optional: Check **Repeat calibration every … sample cycles** and enter an interval to repeat the calibration curve at the specified interval throughout the assay.

Check **Run last** to include an additional calibration cycle at the end of the assay. This will ensure that calibration trends can be used correctly in the Evaluation Software to compensate for drift in calibration responses during the assay. Enter the D-antigen concentration for each point in the calibration curve (*see* Table 1 for D-antigen concentrations of International Reference Pu91-01). You can change the concentration unit for the analyte samples in the table header. Click **Next**.

8. The window **Control Samples** appears. Skip the **Control Samples** window and add the control vaccine samples to the sample table (*see* **Note 13**). Click **Next**.
9. The **Sample Table** appears together with a **Sample and Position Import table**. Enter cancel. Enter the sample names and an optional dilution factor (*see* **Note 14**). The dilution factor is used to calculate the concentration in the original sample (e.g., if you enter a dilution factor 5, a measured concentration of 3 μM in a sample will be reported as a calculated concentration of 15 μM). If you leave the dilution factor blank, a value of 1 will be used. Each row in the table represents one cycle. If you want to run replicate samples, enter the same sample name on different rows. Click **Next**.
10. The window **System preparations** appears. Tick off **Prime before run**. Enter **analysis temperature**, 25 °C, **compartment temperature** 25 °C. Press **Next**.
11. The window **Rack positions** appears. This dialog box shows where samples and reagents are placed in the microplate and rack (*see* **Note 12**). You can change sample and reagent positions manually: Click on the sample or reagent in the sample plate and rack illustration and drag it to a new (empty) position. You cannot drag to a position that does not have sufficient capacity for the required volume of sample or reagent. Positions can also be reorganized using **Automatic positioning** under the **Menu** button (*see* **Note 15**).
12. Prepare samples, mabs, calibration curves, and controls (*see* Subheading 2.1, **items 7–9**) in the required volumes and concentrations (*see* **Note 16**) and place them in the appropriate positions according to the dialog box. If necessary, eject first the required rack from the T200.
13. Click again **Eject rack**. The rack compartment opens. Place the rack into the compartment and click **OK.** Click **Next**.
14. A window **Prepare Run Protocol** appears. Perform the instructions in the dialog box (*see* **Note 10**). Click on **Save as** and enter a name for the concentration method. Click on **Run** and enter a file name for the result file.
15. The sensorgram window appears. This window displays the sensorgram in real time for the current run. Figure 7 shows an example of a complete run.

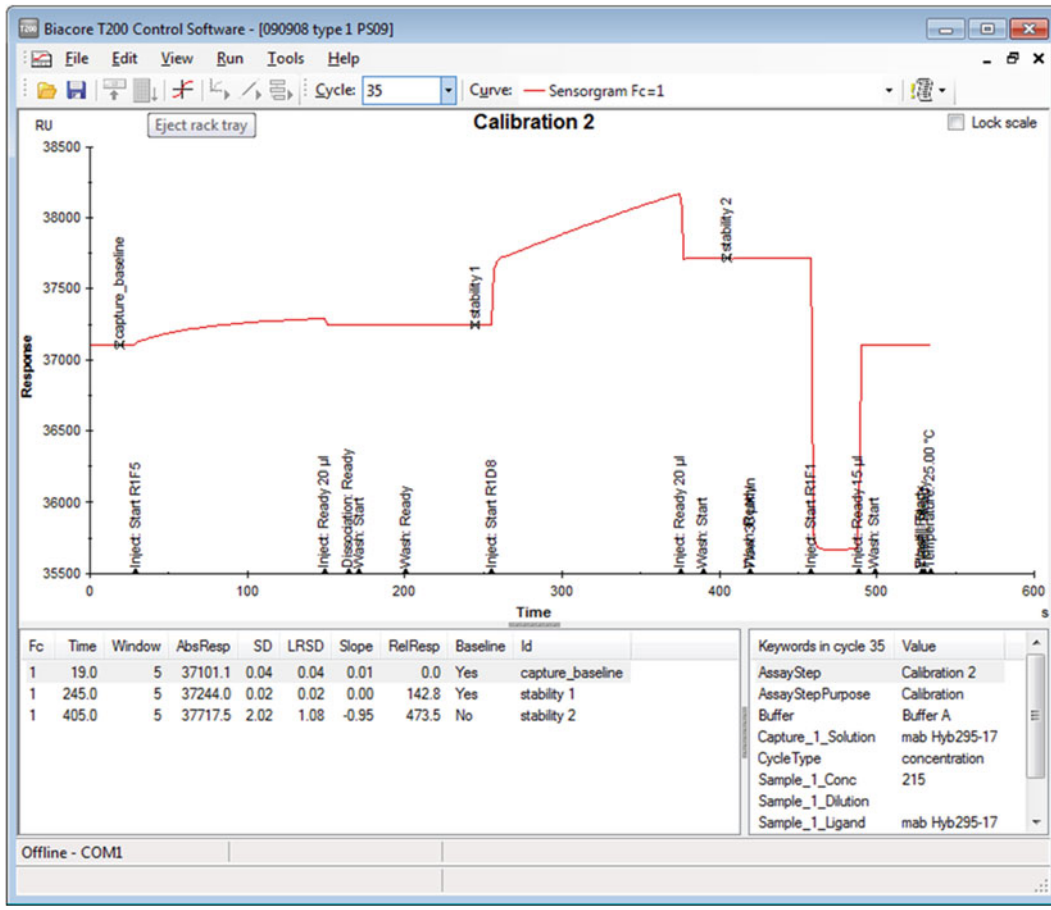

Fig. 7 Example of a sensorgram calibrated D-antigen assay

16. After the run is finished, the Biacore T200 Evaluation software starts automatically and shows an overlay of all generated sensorgrams (*see* Fig. 8) (*see* Subheading 3.4) (*see* **Note 17**).

3.3 Calibrated D-Antigen Assay for Two or More mabs and/or Serotypes in One Method

Because the same trivalent International Reference will be used in one method to quantify the D-antigen amount of two or three serotypes sequentially, it is necessary to build up a method in which the calibration curve can be allocated to a specific serotype. The concentration wizard for the calibrated D-antigen assay for one serotype can be used as base for the method builder.

1. Open the Biacore T200 control software.
2. Click **File**, then **Open/new Method**. Check **Open importable wizard templates** and **browse** to the calibrated D-antigen assay, e.g., "concentration assay type 1 .blm" (*see* **Note 18**).
3. Because the instrument settings are already imported by importing the concentration assay method, we can skip the general settings. Click **Assay steps** from the main menu **Method Builder**.

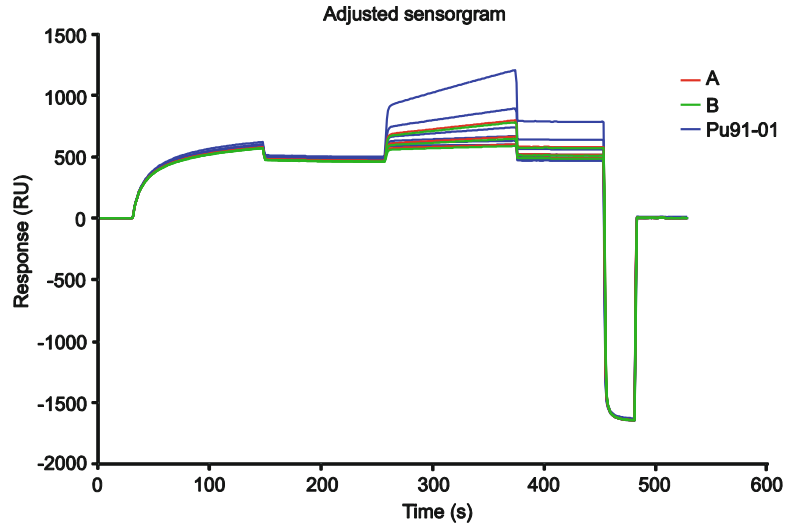

Fig. 8 Overlay of sensorgrams calibrated D-antigen assay with International Reference Pu9-01 and two vaccines A and B

4. Change the name **Calibration** in calibration 1 by clicking at the name in the flow diagram. The name appears in a window in the base settings where you can edit the name. Change also the name **Sample** in Sample A.
5. Copy in the flow diagram the two parameters **calibration 1**, and **sample A**. The flow diagram is now extended with two copies of the parameters. Change the names of these parameters in **calibration 2**, and **sample B** (*see* Fig. 9). Repeat instruction 5, if necessary and feasible (*see* **Note 12**), for the third serotype.
6. Click **Cycle type**. Cycle types define the series of injections and associated parameters that will be used in a cycle. Because we have imported the concentration assay method, the concentration cycle type is already available. Delete **Conditioning** (or any other cycle types) and add **Start up**. Define for Start **up** the series of injections: (1) On the **Commands** tab, select the command **Sample** from the drop-down list and click **Insert** to add the command to the cycle type definition. Next, specify the **Settings** for Sample 1 in the right-hand panel: First unclick in **Method variables** all possible variables. **Type** low sample consumption, **Sample solution** HBS-P, **Contact time** 120 (s), **Dissociation time** 0 (s), **Flow rate** (5 µl/min). (2) On the **Commands** tab, select the command **Regeneration** from the drop-down list and click **Insert** to add the command to the cycle type definition. Next, specify the **Settings** for Regeneration 1 in the right-hand panel: First unclick in **Method variables** all possible variables. **Type Regeneration solution** Glycine-HCl, **Contact time** 30 (s), **Flow rate** (30 µl/min).

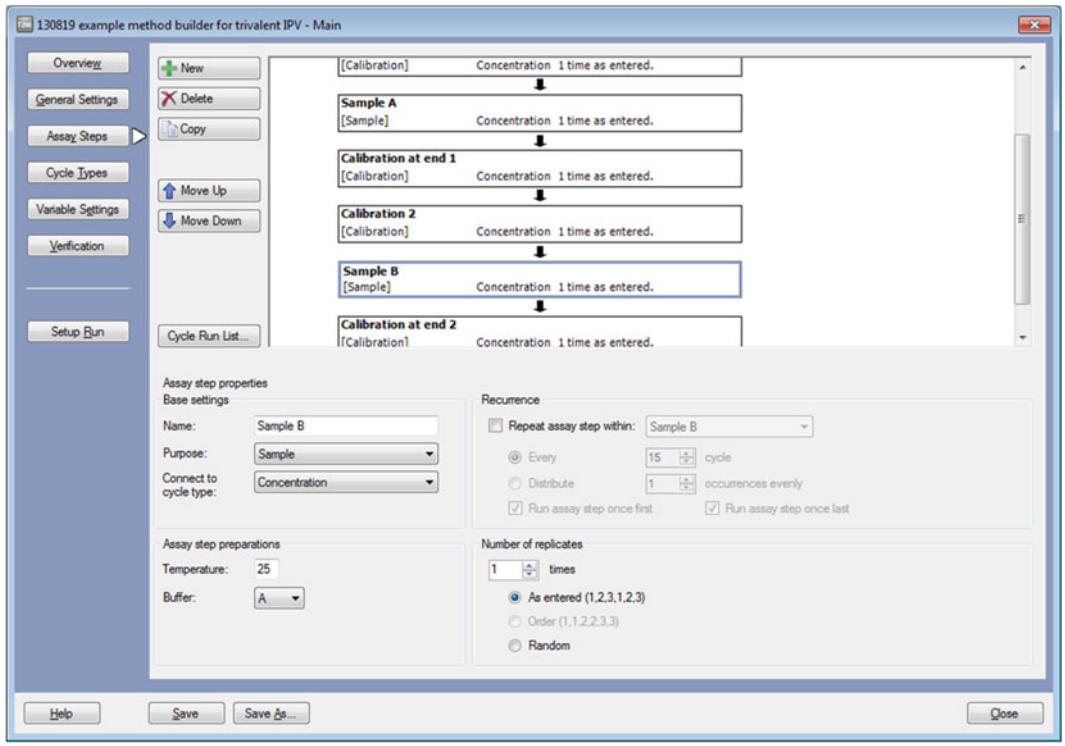

Fig. 9 Example of Assay steps in the method builder for a calibrated D-antigen assay with two or more mabs and/or serotypes

7. Check the **settings** of the **concentration cycle type**: **Capture 1: Capture solution:** variable (check **Method variables**), **Contact time** 120 (s), **Flow rate** (10 µl/min); **Sample 1: Type** low sample consumption, **Sample solution** variable (check **Method variables**), **Contact time** 120 (s), **Dissociation time** 15 (s), **Flow rate** (10 µl/min); **Regeneration 1: Type Regeneration solution** Glycine-HCl, **Contact time** 30 (s), **Flow rate** (30 µl/min).
8. Click **Variable settings**. Click in the left-hand panel assay step **Start up**, check in the right-hand panel, **define all values in method**. Repeat this for each assay step. Next, click in the left-hand panel assay step **Start up**. Enter in row **1**, both columns **Capture solution** and **Solution** HBS-P. Click the next assay step **Calibration 1**. Enter in column **Capture solution** (in case of serotype 1) 8× Hyb295-17 (1 per row), column **Solution** 8× Pu91-01, and column **Concentration** 8 D-antigen concentrations of Pu91-01 (*see* Table 1) (*see* **Note 19**). Next, click assay step **Sample A**. Enter **Capture solution, Sample solution** and **Dilution** (of the sample) in the appropriate columns. Please note that the capture solution should always be the same solution as the capture solution for

the corresponding calibration curve, e.g., the mab against type 1. Control vaccine samples can be added to the Sample A table. Repeat the steps of calibration 1, and sample A, for calibration 2, and sample B. Please enter for all assay steps the same capture solution, e.g., the mab against type 2. Control vaccine samples can be added to the Sample B table. If there is enough room for a third series (*see* **step 5**), repeat the steps for calibration 3, etc. The capture solution for this series might be the mab against serotype 3 (*see* **Note 20**).

9. Click **Verification**. This workspace reports the results of method verification. If the method is correctly defined and parameters are entered where required, the message is: "The method has been verified and can be used to set up a run". If an error in the method occurs, the verification window will indicate which step is mistaken and how to solve this.
10. Click **Set up run**. Select the appropriate **flow path**. Click **Next**. The cycle run list appears. This workspace summarizes the cycles that will be run in the entire method. Use this information to check that the method is correctly defined, that the cycles are run in the intended order, and that variable values have been correctly entered. Click **Next**.
11. The window **System preparations** appears. From here, follow steps **10–16** of Subheading 3.2.

3.4 Biacore T200 Evaluation Concentration Assay

After the method run is finished, the evaluation software starts automatically (*see* **Note 17**). At first sight we see an overlay of all the sensorgrams depicting all cycle runs.

1. Check the overlay on irregularities, e.g., air spikes, drifting baselines. Identify which cycle causes the irregularity: Click **cycle <overlay>** and click cycles one by one (*see* **Note 21**). If an irregularity has occurred in one of the cycles, take that into consideration at the concentration analysis evaluation (e.g., skip the results of this specific cycle, *see* **Note 24**).
2. In the menu bar, click **Concentration analysis** and then **Using calibration**.
3. Choose the **Flow cell** (the immobilized flow cell), **report point** (stability 2; this is the response after binding of the sample to the capture antibody), (*see* also Fig. 10) and Response type (relative response) to be used for the calibration curves. All calibration curves in the evaluation item use the same settings.
4. Calibration curves are shown in the right-hand panel. Use the browse buttons or the selector button at the top of the panel to select which calibration curves to display. The available options depend on the way the calibration curves are used: for the Use average calibration curve option a single average calibration curve is displayed (*see* **Note 22**).

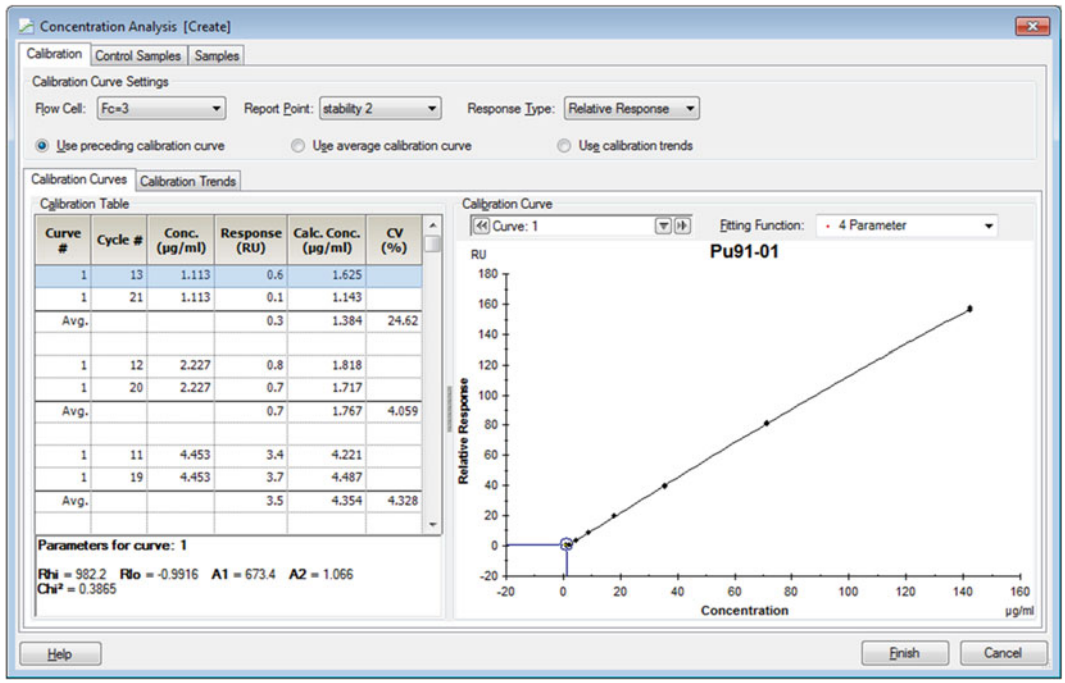

Fig. 10 Example of the BIAevaluation concentration analysis window

5. Choose the 4-parameter fitting as the **fitting function** for the calibration curve (*see* **Note 23**).
6. Click for sample and control results the relevant tabs. The **Sample tab** shows the results of concentration analysis for the unknown samples (*see* Fig. 11): Sample data is listed in the left-hand panel. The Calc.Conc. column in this table shows the measured concentration multiplied by the dilution factor (i.e., the concentration in the original sample before dilution). Concentrations are calculated according to the setting on the Calibration tab. The calibration curve used to calculate sample concentrations is listed in the Calib.Curve column. Samples that lie outside the range of the calibration curve are listed as above or below the limits of calibration as appropriate. The right-hand panel shows the calibration curve used for the currently selected sample. Calibration points are shown in red and samples in black (*see* **Notes 24** and **25**).
7. Click **Finish** to save the concentration analysis evaluation item.

3.5 Calibration-Free Concentration Analysis

This assay needs monoclonal antibodies that have a high affinity to polio epitopes (*see* **Note 26**). The mabs, mentioned in the materials, meet this criterion. The vaccine samples should be diluted to 0.5–1 µg/ml protein in HBS-P, mabs can be diluted 250× in HBS-P. For this method we also need the molecular weight of a polio particle (8250 kDa) and the diffusion coefficient (D) of poliovirus

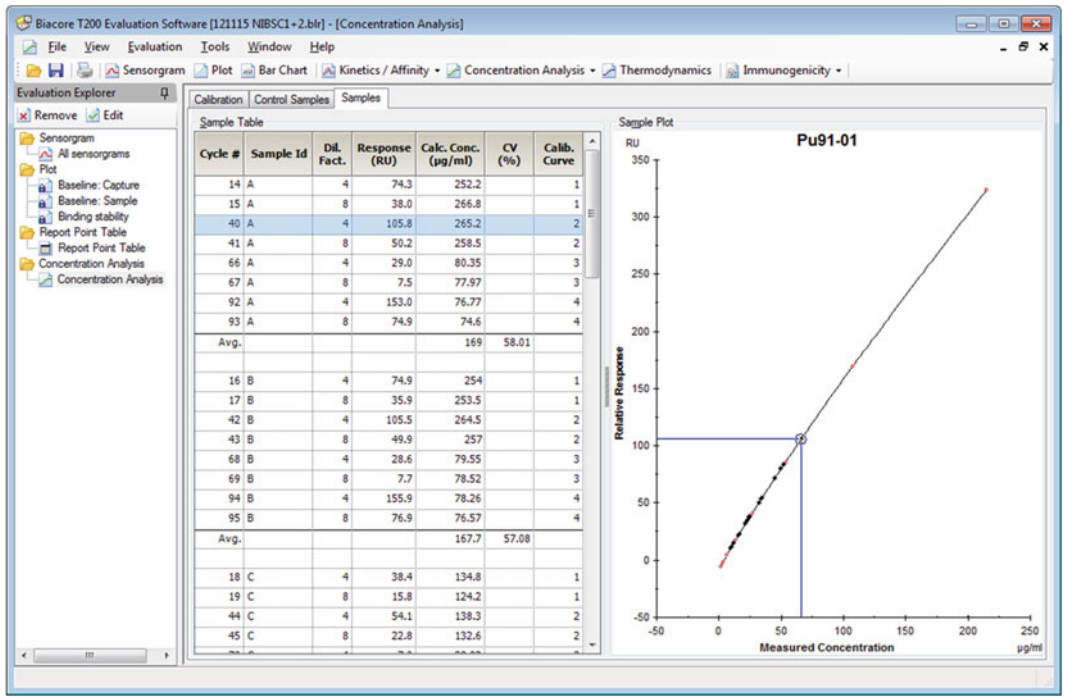

Fig. 11 Example of the D-antigen concentration evaluation

(1.44E–11 (m^2/s) at 20 °C) [11]. A CM3-sensorchip is used for the immobilization; however, besides the immobilization of Goat-anti mouse IgG Fc-antibodies, a blank immobilization of a second flow cell is needed. This flow cell serves as a reference flow cell. Regarding the immobilization procedure, *see* Subheading 3.1.

1. Open the Biacore T200 control software.
2. Click **File**, then **Open/new Method**. Click in directory **Biacore Methods** Calibration-free concentration (*see* **Note 27**). Click **Open**.
3. Click **General Settings**; choose **Concentration Unit** µg/ml.
4. Click **Assay Steps**; choose **Startup**, then enter **Number of replicates** 5.
5. Click **Blank**, then enter **Recurrence every** ten **cycle.**
6. Click **Cycle Types**, and then click cycle types **Sample**. On the **Commands** tab insert the command **Capture**. The sequence of commands for cycle type **sample** is: **Capture 1, Sample 1, Regeneration 1**. Next, specify the **Settings** for Capture 1 in the right-hand panel: First click in **Method variables** capture solution. **Contact time** 60 (s), Flow **rate** (10 µl/min), **Flow path** both.
7. Click **Variable settings**. Click **sample** in **Assay steps**. Delete "No" in the **Sample1 Blank** cell (the cell ought to be empty).

Table 2
Example of variable values for assay step "sample" in method builder Calibration-free Concentration assay (*see* Subheading 3.5 step 9)

Capture solution	Sample solution	Flow rate (μl/min)	M_W (Da)	Diffusion coefficient (20 °C)	Dilution
mab 295-17	A	**5**	8,250,000	1.44E−11	10
mab 295-17	A	**100**	8,250,000	1.44E−11	10

8. Click **Verification.** This workspace reports the results of method verification. If the method is correctly defined and parameters are entered where required, the message is: "The method has been verified and can be used to set up a run". If an error in the method occurs, the verification window will indicate which step is mistaken and how to solve this.
9. Click **Set up run.** Select the appropriate **flow path** (either **2-1** or **4-3**, dependent of which flow cells are immobilized for this assay). Click **Next.** A **Sample and Position Import table pops up**. Click Cancel. Enter the variable values for **Assay step Sample** (Table 2). Note that the variables must be entered in duplicate because of the two different flow rates.
10. Enter the variable values for **Assay step Blank in** row 1 and 2: buffer. Click **Next**.
11. The cycle run list appears. This workspace summarizes the cycles that will be run in the entire method. Use this information to check that the method is correctly defined, that the cycles are run in the intended order, and that variable values have been correctly entered. Click **Next**.
12. The window **System preparations** appears. From here, follow **steps 10–15** of Subheading 3.2.

3.6 Biacore T200 Evaluation CFCA

After the method run is finished, the evaluation software starts automatically (*see* **Note 17**).

1. In the menu bar, click **Concentration analysis** and then **Calibration free**.
2. A window **Select samples** appears. Click on the checkmarks for **Expand all cycles**, **Show original sensorgrams**, and **Use reference subtracted data.**
3. Choose which samples to evaluate: The table in the top panel lists all samples that can be evaluated. Remove the checkmark from a sample row to exclude the sample from the evaluation, or from a cycle row to exclude the individual cycles. Use the

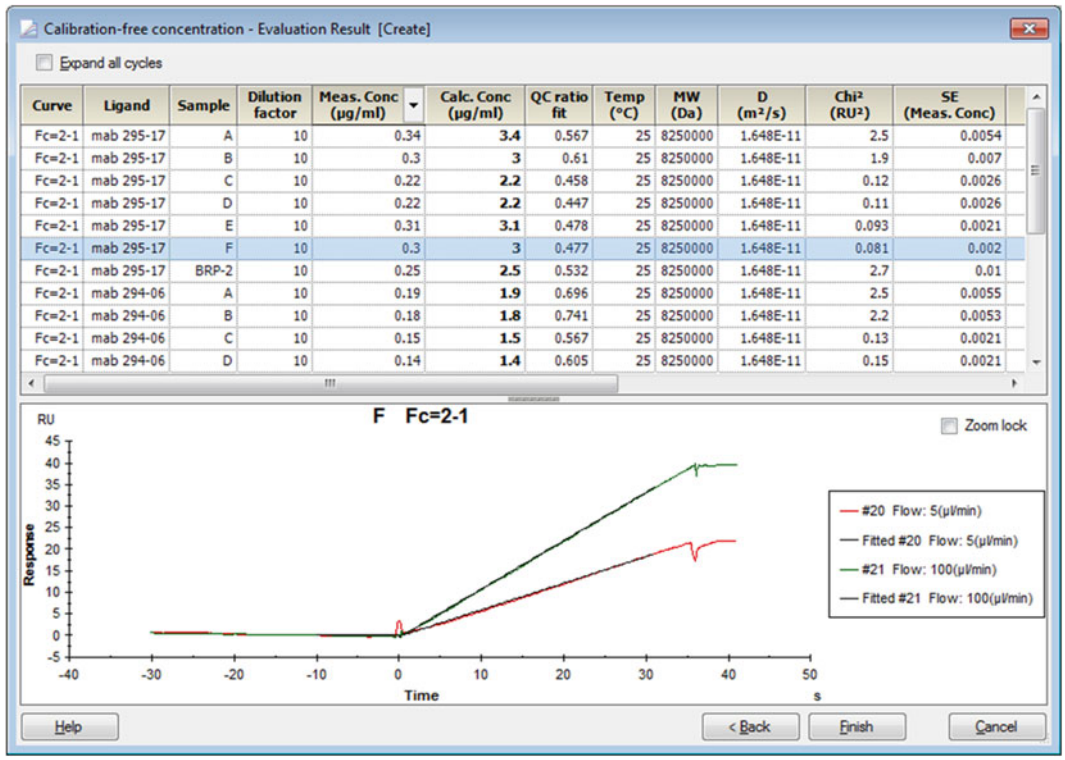

Fig. 12 Example of the calibration-free concentration assay evaluation

QC ratio (>0.20), Initial rate (<0.3 RU at Flow = 5) and the sensorgram appearance to help in deciding whether to exclude samples (*see* **Note 28**). Click **Next**.

4. This step shows the results of the evaluation. The results are presented as a table of samples with calculated concentration values (*see* Fig. 12). Use the **Expand all cycles** option to show or hide cycle details for each sample. Concentrations are reported as values measured in the injected sample (Meas. Conc) and values calculated for the sample before dilution (Calc. Conc = Meas. Conc × Dilution factor). Choose the unit for reported concentration in the column header for **Meas. Conc**.

4 Notes

1. A CM3 chip is recommended (instead of the regular CM5 chip) because an (inactivated) poliovirus particle is large (±30 nm) compared to individual protein molecule. The shorter dextran chains on the CM3 chip allow the interaction to take place closer to the surface which can improve sensitivity.
2. Because EDC is sensitive to hydrolysis, it is important to freeze the aliquots of EDC immediately.

3. Each laboratory will have an own panel of monoclonal antibodies. Especially for serotype 3, it is important to choose a mab that is site 1 specific because of a possible trypsin-induced degradation of site 1 which might be overseen if a site 2 or 3 specific mab would have been used.
4. The D-antigen concentration should be above the lower limit of quantification of 3-0.4-1.1 DU/ml for type 1, 2, and 3, respectively.
5. Although these tubes do not have the same size as micronic tubes, they do fit in a micronic rack, which makes it easier to handle a multichannel pipette.
6. The method is also suitable for Biacore T100 software.
7. To get rid of unimmobilized molecules and to stabilize the base line after immobilization faster, we adapted the amine coupling method by adding three injections of regeneration buffer: in the immobilization set-up window, press **Custom Methods**. A new window **Custom Methods** appears. Click from the methods **Amine**. Press **Copy**. In the methods window **Copy of amine** appears. Add to the command list (lower window) **Inject** by pressing the **inject button.** In the new window fill in **solution**: Glycine-HCl, **contact time** 30 (s), **flow rate 5** (μl/min). Press **Add**. Repeat this inject **command** twice. Change the **method name** in "Amine + regeneration 3×". Press **OK**. The new immobilization method is now added to the Method scroll menu in the immobilization set up window (Fig. 13).
8. You can change the reagent rack and microplate types in the drop-down lists above the respective illustrations. If you do so, all positions in the affected rack or plate will be cleared and must be reassigned either manually or automatically.
9. Solution volumes listed in the table are minimum volumes unless they are specified as **Exactly.**
10. The estimated run time and buffer consumption are minimum values. Estimated buffer consumption is calculated with a dead volume of 50 ml in the bottle and rounded up to the nearest 100 ml. We recommend diluting the 10× HBS-P buffer (running and dilution buffer for both immobilization and D-antigen c assays) to a volume of at least 1 l, to have enough for the concentration assays as well.
11. Immobilization levels to be expected are between 4000 and 6000 RU.
12. It is advisable to use Rack 1 in combination with a microplate. In this setting one serotype could be measured in one run with a maximum of 26 samples in three dilutions or in case of three serotypes (*see* also Subheading 3.3), 16 samples in three dilutions.

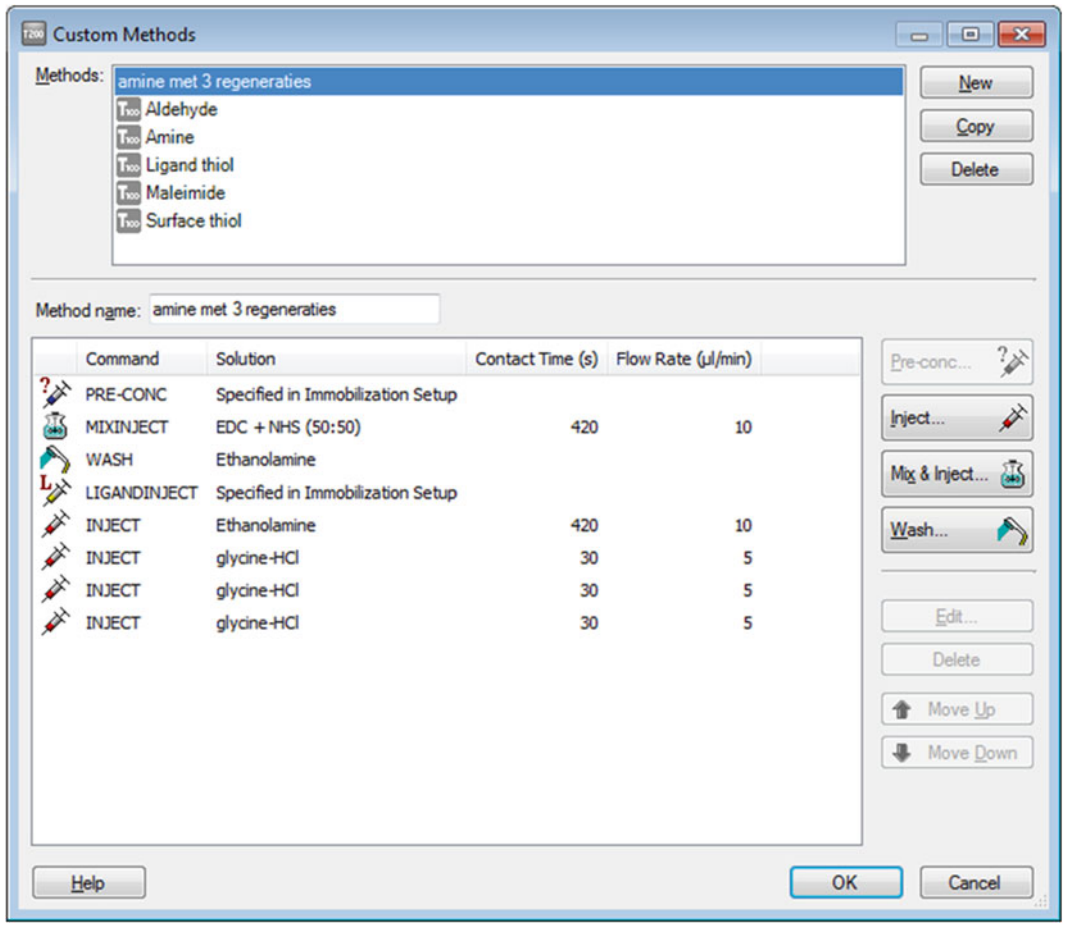

Fig. 13 Command for customized Amine method: Amine coupling with three regenerations

13. If you prefer to present control samples separately you may check **Run control samples**. Control samples are presented separately in evaluation of concentration analysis. Check **Repeat control sample(s) every … sample cycles** and enter an interval to repeat the control samples at the specified interval. Controls will be run at the start and end of the assay and at the specified interval. If this option is not checked, controls will be run once at the start of the assay. Enter sample names and expected concentrations for the control samples. Measured concentrations for control samples are expressed relative to the expected concentrations in the evaluation. The unit for expected concentrations is the same as that chosen for the calibration curve. Concentrations for control samples should be within the range covered by the calibration curve, to allow the measured concentrations to be determined. Each row in the table represents one cycle. If you want to run replicate control samples, enter the same sample name on different rows.

14. Although it is optional, we recommend a twofold dilution series of three dilutions in HBS-P within the calibration curve range.
15. If using a microplate and a multichannel, it is more convenient to place the dilution series of calibration curves and samples in rows of descending order. Click the **Menu** button and the **Automatic Positioning**. Choose for the **Calibration** and **Samples** the row **Orientation, Anchor** bottom left and **First Sort** by content descending. Check if by choosing a larger **Vial Size** and **Pooling** yes, the content of two or more vials for one solution can be merged. Click **apply**.
16. In case of using other mabs than mentioned in the materials section, find a mab dilution that generates a signal above 100 Response Units (RU) after capturing the International Reference in the 1:1 dilution. This can be explored by the **Binding Analysis** wizard. Follow the instructions in Subheading 3.2, **steps 3–6**, but instead of checking the **Ligand Capture**, check **Sample** [2]. In the window **Injection Parameters**, the **Ligand Name** and **Sample** are replaced by **First Sample and Second Sample**. Enter in the sample table into the sample ID 1 column: mab A in a different dilutions, e.g., row 1 mab A 1:100; row 2 mab A 1:500; row 3 mab A 1:1000. Enter in column Sample Id 2 Pu91-01 (diluted 1:1) 3× in row 1, 2, and 3, respectively. Click **Next** and follow instructions according to Subheading 3.2, **steps 10–16**.
17. The evaluation software can also be started separately from a specific method.
18. It is easier to import a wizard that has been made for a calibrated D-antigen assay for one serotype and one mab.
19. Right-click on a row in the table for options for copying and pasting variable values and for inserting and removing rows.
20. Instead of capture mab solutions of serotype 1, 2, and 3, you may use two or three different site specific mabs for the same serotype. In this case, the purpose of the assay is not just D-antigen quantification but also epitope characterization. However, the capture mab solution should always be the same for both sample and calibration curve.
21. If you block the calibration curve cycles in the **cycle<overlay>**, the overlay will show just these cycles. By blocking certain cycles you will get a good impression of the quality of the cycle runs.
22. In the **calibration curve settings** different options for using calibration curves are available: **Use preceding calibration curve, Use average calibration curve**, and **Use calibration trends**. Normally the use of an average calibration curve is recommended, but in case of a single calibration curve of an increasing baseline it is better to choose the **Use preceding calibration curve** option.

23. If the calibration curve shows an obvious outlier, you may choose to exclude this point: right-click on a point in the plot or on a row in the calibration data table and choose Exclude cycle to exclude the point from the fitting. Excluded points are shown with open symbols. Right-click on a curve and choose Exclude curve to exclude the calibration curve. Excluding a curve automatically excludes all the calibration points in the curve. If you reinclude sufficient points to allow calibration curve fitting, the curve will still be excluded but will be shown as a broken line.
24. If a sample point shows an obvious outlier (*see* also Subheading 3.4 **step 1**) and you want to exclude this point: Right-click on a point in the plot or on a row in the sample data table and choose Exclude cycle to exclude the sample from the average and CV calculations. Excluded points are shown with open symbols.
25. In case of the calibrated D-antigen concentration assay with two or more mabs and/or serotypes, be aware that average and CV calculations in the sample data table are based on the results of one sample and could be derived from more than one calibration set, e.g., in case of a trivalent sample the average result could be the average D-antigen concentration of serotype 1, 2, and 3. In this case it is necessary to calculate average and CV per calibration curve.
26. The association and dissociation rates of the used mabs are unknown; however, these mabs show high QC ratios. Using a low affine mab translates into overlapping initial binding rates at the two different flow rates which results in a too low (<0.2) QC ratio.
27. The CFCA assay is included in the regular Biacore methods and contains a lot of default settings in the method builder. With regard to polio vaccines we have made a few adjustments to the method and only these adjustments will be dealt with.
28. The QC ratio provides an indication of the extent to which the initial binding rate is influenced by flow rate. The QC ratio in the results table is calculated from the fitted curves and not from the original sensorgram data. In this way, disturbances and excluded data in the original sensorgrams do not affect the reported QC ratio, except insofar as they affect the fitting. Exclude samples where the QC ratio is lower than about 0.2 and where the initial binding rate at the lower flow rate is too low (<0.3 RU) to be reliably measured. Check also the sensorgram appearance. Exclude cycles that are obviously disturbed. The sensorgram at the lowest flow rate should be approximately linear over the first 30 s of sample injection. Exclude samples where the sensorgram at the lowest flow rate

shows excessive curvature. Note that if you exclude individual cycle from a sample you have to exclude also the cycle with the second flow rate because analysis of each sample uses at least two different flow rates.

References

1. Wood DJ, Heath AB, Kersten GF et al (1997) A new WHO International Reference Reagent for use in potency assays of inactivated poliomyelitis vaccine. Biologicals 25:59–64
2. van Steenis G, van Wezel AL, Sekhuis VM (1981) Potency testing of killed polio vaccine in rats. Dev Biol Stand 47:119–128
3. Bevilacqua JM, Young L, Chiu SW et al (1996) Rat immunogenicity assay of inactivated poliovirus. Dev Biol Stand 86:121–127
4. Guideline on waiving the *in vivo* assay of poliomyelitis vaccine (inactivated) and its combinations. In: European Pharmacopoeia. 2720 7th ed: EDQM
5. Morgeaux S, Milne C, Daas A (2005) Feasibility study to develop a common in vitro D antigen assay for inactivated poliomyelitis vaccines. Pharmeuropa Bio 2005:19–26
6. Westdijk J, Brugmans D, Martin J et al (2011) Characterization and standardization of Sabin based inactivated polio vaccine: proposal for a new antigen unit for inactivated polio vaccines. Vaccine 29:3390–3397
7. Wood DJ, Heath AB, Sawyer LA (1995) A WHO collaborative study on assays of the antigenic content of inactivated poliovirus vaccines. Biologicals 23:83–94
8. Bakker WA, Thomassen YE, Van't Oever AG et al (2011) Inactivated polio vaccine development for technology transfer using attenuated Sabin poliovirus strains to shift from Salk-IPV to Sabin-IPV. Vaccine 29:7188–7196
9. Biacore concentration analysis handbook, BR1005-12 Edition AB/01-2007, GE Healthcare
10. van der Merwe PA (2001) Surface plasmon resonance in protein-ligand interactions. Oxford University Press, London
11. Koch F, Koch G (1985) The molecular biology of poliovirus. Springer-Verlag Wien, New York