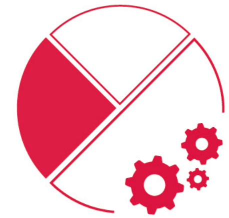
FOx BIOSYSTEMS
Breaking limits in bioanalysis



WHITE PAPER

Carboxyl probe immobilization for label-free protein quatification

White paper 1 | Version 2 | Filip Delport, Dagmara Minczakiewicz, Kris Ver Donck

Abstract

This white paper describes a general strategy for optimizing immobilization on the FO-SPR probes and provides some reference example data as comparative guidance.

Surface plasmon resonance (SPR) is a powerful technique used to study a wide variety of label-free biomolecule interactions. However, there is no 'one size fits all' solution for ready-to-use applications and consumables that matches the variety of biomolecules used. Here, we describe a methodological approach to configure your specific biosensing surface on the generic carboxyl SPR probe for quantification purposes.

With this stepwise procedure, you can optimize the immobilization of a capture molecule, such as a specific monoclonal antibody, to obtain reproducible biosensors for quantifying your target antigen. This approach can be extended to other capture and target biomolecules, since SPR provides sensitive, label-free detection, irrespective of the type of binding interaction.

The flexibility to set up protocols and modify individual parameters from previously-used, stored protocols makes it easy to screen multiple condition variations, while real-time data visualization provides immediate feedback on your tests.

Furthermore, the unique FO-SPR probes can be dipped into the sample in a non-destructive method which only requires low volumes of precious samples which can be completely recovered afterwards, and also avoids clogging, thereby overcoming the limitations of microfluidic systems.

Introduction

To measure or quantify the amount of a specific protein in a sample using an SPR sensor, you need a sensor surface that can selectively bind the target protein in a dose-dependent manner.

Starting from the general-purpose carboxyl surface probe, available as an off-the-shelf product, you will need to effectively and efficiently immobilize a capture molecule to the carboxyl surface. This capture molecule is typically a protein, and often a specific antibody against the target protein of interest. To achieve reproducible target molecule quantification, you need to run a series of optimization experiments to identify optimum conditions for forming a reproducible surface immobilization, and consequently, target capture and quantification.

Keep in mind that there is no 'one size fits all' solution in SPR surface immobilization. Every pair of capture and target molecule behaves differently and may require its own optimized protocol. Also, the optimization for target quantification may differ from optimizing other parameters like studying binding kinetics, even for the same capture molecule.

If you follow this optimization strategy and adjust it to your needs, you will be able to identify optimal, reproducible immobilization conditions for your capture molecule for target quantification.

Materials and Methods

What is FO-SPR

FOx BIOSYSTEMS turned an optical fiber into a mass-sensitive sensor using the well-established surface plasmon resonance (SPR) principle for biomolecular interaction analysis.

The SPR effect is achieved by coupling a white light source to the fiber optic sensor probe. In this consumable probe, the light interacts with a gold layer and senses the refractive index up to 200 nm away from the outer surface. At the end of the probe, light is reflected back through the bifurcated fiber to a spectrometer. The resonance condition is monitored by tracking the wavelength at which the least light is reflected.

The fiber optic surface plasmon resonance (FO-SPR) sensor is coated with bioreceptor molecules which can bind to the target molecules of interest. This binding changes the refractive index resulting in a wavelength shift that produces a sensor signal. The sensor probe is simply dipped into the liquid sample to measure biomolecular interactions directly.

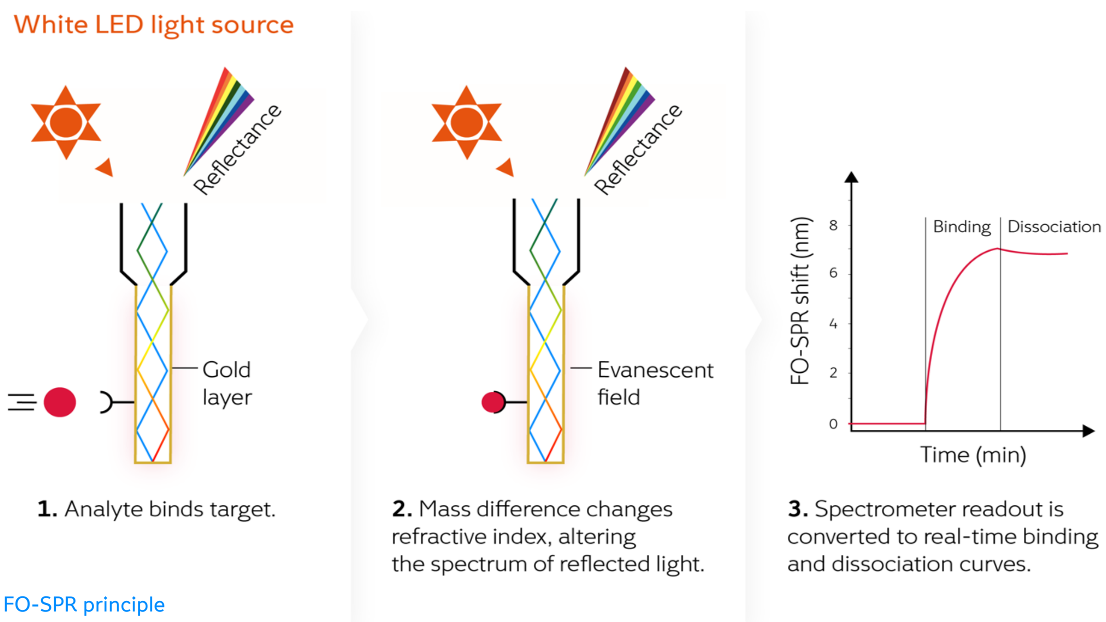


Figure 1: FO-SPR principle

Tools and reagents

For the procedures in this white paper you will need:

- White FOx instrument with FOx-SPR acquisition software and the FOx data processing tool
- Carboxyl probes for label-free quantification (FOx BIOSYSTEMS product nr: 30.0003)
- Microsoft Excel or other data calculation software for data handling and viewing
- Micro pipettes from 10 to 1000 µl with disposable tips
- A 96 well PCR plate or 8 well PCR strips
- On-desk refrigeration for protein solutions

Most protocol steps are run with commercially-available, off-the-shelf reagents and buffers. pH ranges are used in the buffer optimization phase to best match the pI value of your capture molecule to be immobilized. Once optimized, you can simply use the optimal buffer and pH.

Buffer/reagent	Concentration	pH (-range)
NaAc: Sodium acetate	10 mM	4.0; 4.5; 5.0; 5.5
MES: 2-(N-morpholino) ethanesulfonic acid	50 mM	6.0
MES: 2-(N-morpholino) ethanesulfonic acid	10 mM	5.5; 6.0; 6.5
HEPES	10 mM	6.5; 7.0; 7.5; 7.5
EDC: 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide	800 mM (fresh) 400 mM in EDC/NHS	50 mM MES pH 6.0
NHS: N-hydroxysuccinimide	200 mM (fresh) 100 mM in EDC/NHS	50 mM MES pH 6.0
Ethanolamine-HCl	50 mM	8.5
Capture molecule to immobilize		

Table 1: Reagents and buffers.

Note: Special attention is required for EDC/NHS which is a highly reactive and short-lived reagent (EDC user guide, Sigma Aldrich) and needs to be prepared just prior to use. Separately dissolve NHS and EDC no more than 5 minutes before use, as listed in Table 1, mix in equal quantities and pipet into the designated wells immediately before use.

Alternatively, you can also prepare pre-dissolved aliquots of EDC and of NHS respectively and store them at -20°C until use. Thaw one aliquot of each at room temperature for about 20 minutes before use. Mix in the designated wells just before placing the reagent in the instrument with your run sequence (see Table 2).

Instrument protocols

All steps involving the carboxyl probes are run on the White FOx instrument, allowing you to monitor the immobilization process in detail and control or adjust protocol steps in subsequent optimization runs. An immobilization protocol for a carboxyl probe typically consists of 3 essential steps:

1. Chemical activation of the probe surface
2. Capture molecule immobilization
3. Deactivation and wash

Before each of these steps, a buffer equilibration will prime the surface for the next treatment and provide a baseline for measuring the changes induced by the reaction. A wash step will then stop the reaction after incubation and prevent carry-over of reagents. Table 2 describes the general immobilization protocol step sequence and suggested probe dwell time.

Both sequence and dwell time may differ between experiments and can be adjusted to be optimal for your capture molecule immobilization. We recommend that you start optimization with a prolonged incubation time, as depicted here, and shorten the incubation afterwards when stable conditions allow. Figure 2 shows a typical long run of an optimized immobilization with clear SPR shift values, saturation of capture molecule binding to the EDC/NHS activated surface, and minimal binding loss after ethanolamine quenching.

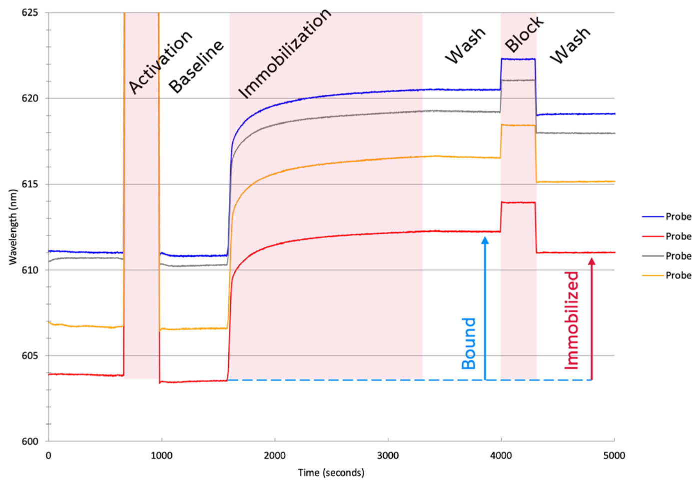


Figure 2: Example sensorgram with an extended immobilization step.

To prepare the process run, check the following steps:

- Turn on the White FOx instrument and laptop and start the FOx SPR software to warm up the instrument (including LEDs) for 1h before use.
- Equilibrate all the buffers and reagents to room temperature (RT) before use. Degas if possible and mix and short spin prior to use. Protein stock solutions should be kept cold, but dilutions for immediate use can also be equilibrated to RT.
- Make sure the instrument run sequence, an example of which is specified in Table 2, is correct and ready. Ensure the run sequence matches the plate reagent layout and applies the correct timing for each step.
- Transfer all the appropriate reagents to the PCR tubes or PCR microtiter plate wells according to the run layout. Ensure that no bubbles are present in the wells. If needed bubbles can be removed by gentle manipulation with a narrow pipette tip.
- Place the tubes or plate in the instrument's plate tubes holder.
- Place the probe rack in the probe pick-up rack holder.

Then close the instrument cover and start the assay sequence run.

Step#	Name	Action	Position	Shaker [rpm]	Time [s]
1	Attach probes	Probe Pick up	A1	-	-
2	50 mM MES pH 6.0	Measure	E2	1000	300
3	EDC/NHS activation	Measure	E2	1000	300
4	Buffer baseline	Measure	E1	1000	300
5	Capture molecule immobilization	Measure	A1	1000	(600) 1800
6	Buffer wash	Measure	A2	1000	300
7	Ethanolamine	Measure	A3	1000	300
8	Buffer wash	Measure	A4	1000	300
9	Collect probes - or continue experiment	Probe Switch			

Table 2: Example run sequence to test for immobilization. Brackets suggest optimized time.

Method development

Label-free quantification of the target molecule can be affected by the details of the method used. Here we describe the optimization method for some critical reagents, buffers and technical parameters for each functional step. Testing one variable at a time will help significantly to save consumables in the optimization phase but may require a few long runs before incubation times are optimized. A good sequence to work through the tests is to:

- First optimize reagents and buffers
- Then optionally test physical parameters like shaking and temperature control
- And finish off by optimizing incubation time

To calculate the (relative) amount of immobilization you can simply calculate the difference between the buffer baseline just before capture molecule binding (internal zero) and the stable signal shift after the blocking step which represents the total immobilized capture molecule (see Figure 2). As an intermediate optimization calculation, you can also refer to the total molecule bound at the shift in wash buffer before blocking, but this intermediate result may still contain some non-specific binding as well.

General protocol

To save on work, we propose using a run with preset activation and deactivation steps and apply fixed shaking and temperature. Optimization will focus on the immobilization step.

For immobilizing IgG antibodies as capture molecules, we typically start with a parameter preset for temperature at 26°C and shaking rotation at 1000 rpm in all incubation steps. The initial timings applied are as listed in Table 2.

The typical final concentrations of EDC/NHS for activating the probe surface are 400 mM and 100 mM in MES buffer pH 6.0 applied over a 5-minute incubation time. For the deactivation step we apply ethanolamine as listed in Table 1 for 5 minutes and wash with the immobilization buffer to calculate the final immobilization shift.

Unless described otherwise, all experiments were performed in quadruplicate, and CV was plotted as error bars.

Reagent and buffer optimization

You can optimize the immobilization buffer by testing a broad pH range using multiple buffers with overlapping pH in increments of 0.5 pH units or less. The best buffers to use depend on the pI of the molecule you want to immobilize and on the pKa of the sensor surface, to create the maximum charge on the surface relative to the bioreceptor for electrostatic attraction.

The best results will be provided by the buffer with a pKa closest to the optimal pH for promoting the reaction.

For IgG immobilization, for example, you can use NaAc buffer from pH 4.0 to 5.5, MES buffer from pH 5.5 to 6.5, and HEPES from pH 6.5 to 8.0. For a final accurate determination run the optimal buffer in replicates for pH values close to the optimum pH from the 1st round. For the data in this white paper we used MES buffer.

Secondly, the capture molecule concentration will affect the duration of the immobilization step. At a fixed pH, test a capture molecule concentration series to assess the optimal binding concentration and deviation.

We can also check if the use of low concentrations of mild detergents on immobilization improves results. Here we tested 0%, 0.01% and 0.05% Tween-20 in the immobilization buffer. Always include the same buffer and detergent concentration in the pre- and post-immobilization wash steps. With this capture molecule, we added 0.01% Tween-20 as a standard immobilization buffer component.

Binding incubation time

For surface activation with prolonged incubation on the immobilization step, we can identify the time to reach the capture molecule binding plateau directly from the sensorgrams, or in post processing from the sensorgram data. Please note, extended incubation time is not always optimal. Reaching plateau will typically be the target in optimizing immobilization for label-free quantification. In this example, we initially ran for 30 minutes and finally fixed immobilization incubation at 10 minutes.

Physical optimization

The instrument is equipped with temperature control and an orbital shaker for the sample plate. Plate temperature and shaking speed can be variably set from the software. We propose running at a controlled temperature of 26°C and applying 1000 rpm shaking. You may want to check alternative conditions from no shaking up to 2000 rpm, and room temperature (RT) or higher, or just run the presets as mentioned here. Please note incubation at temperatures elevated above 30°C does not typically improve immobilization results.

Results

The general immobilization protocol from Table 2 results in sensorgrams as depicted in Figure 2. It is useful to maintain this elongated incubation time for each step when starting the optimization process, to clearly identify plateau phases and stable readouts. You can always zoom in to early binding or dissociation events when changing reagents or buffers.

We generally recommend starting with shaking at 1000 rpm, temperature set to 26°C, EDC/NHS activation for 5 minutes with the concentrations in Table 1, and first focus on identifying the optimal pH of the immobilization buffer, using extended incubation.

As shown in Figure 2, we determined the amount of protein immobilized after quenching with ethanolamine because, for this protein (an IgG antibody), the ethanolamine step quenches the reaction and removes non-covalently bound protein from the probe surface. Alternatives for quenching can be found in the literature if needed.

EDC/NHS activation time and incubation

Figure 3 shows an experiment to verify EDC/NHS activation time and immobilization time. From these experiments, we selected 5 minutes activation time, as longer activation yielded lower immobilization and higher CVs (see Figure 3, right). The immobilization plateau was reached after about 10 minutes at room temperature as shown in Figure 3, left. Although a small increase over time after 10 minutes immobilization is observed, this may allow more non-specific events to take place as well. Therefore, prolonging immobilization time beyond the start of the plateau phase may ultimately result in less optimal performance.

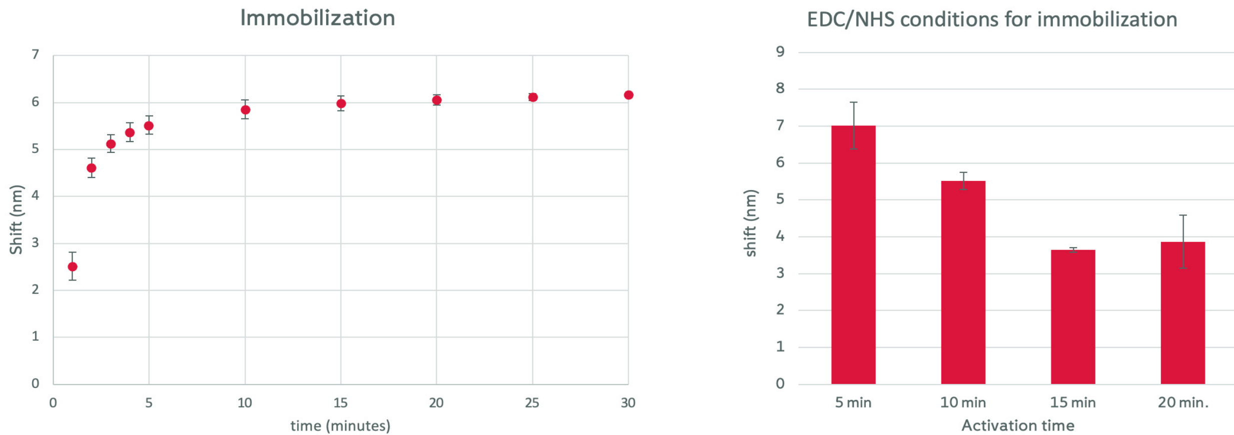


Figure 3: Left - immobilization over time after 5 minutes 400 mM EDC + 100 mM NHS activation. Right – 5 minutes of immobilization following different activation schemes with 400 mM EDC + 100 mM NHS. Tests in duplicate at 1000 rpm at room temperature.

Buffer, pH, and detergents

Buffer comparison with multiple pH points showed MES buffer near pH 5.5 to give the largest shifts with the least variability at pH 5.7, as shown in Figure 4. The optimum concentration of target molecule was reached at 15 µg/ml, as shown in Figure 5. Further refinement using MES buffer with detergents provided the best results and highest reproducibility in Figure 6. Detergent testing indicated the addition of 0.01% tween-20 to the buffer provided the greatest improvement in immobilization, as detailed in Figure 6 and Table 3.

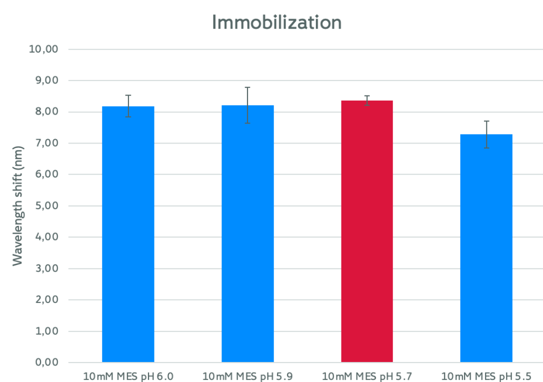


Figure 4: Immobilization at multiple pH conditions. Tests run in quadruplicate.

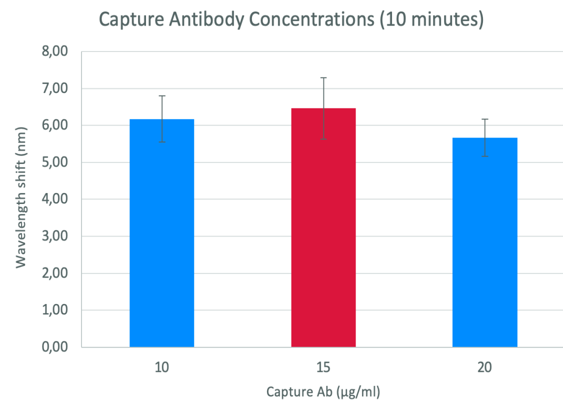


Figure 5: Concentration of capture molecule on immobilization. Tests run in quadruplicate.

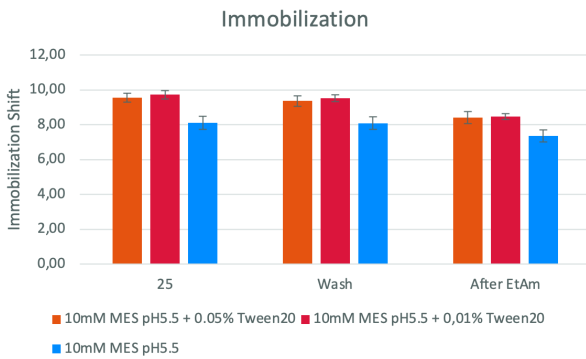
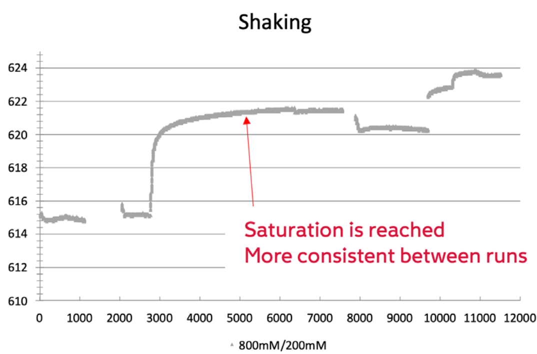


Figure 6: Immobilization in MES pH 5.7 buffer with multiple detergent concentrations at end of immobilization step, after buffer wash and after ethanolamine quenching. Tests run in quadruplicate.

Buffer	Average	StDev	CV%
10 mM MES pH5.5	8.42	0.35	4.10
+ 0.01% Tween-20	8.48	0.17	2.00
+ 0.05% Tween-20	7.37	0.34	4.60

Table 3: Immobilization results after ethanolamine quenching, in MES pH 5.5 buffer with multiple detergent concentrations. Tests run in quadruplicate.

Shaking and temperature



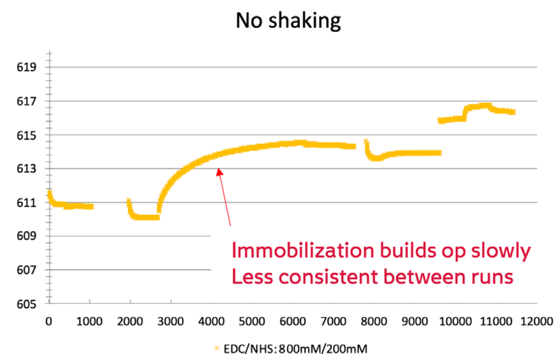


Figure 7: Effect of shaking on the protein immobilization step. Left: 1000 rpm. Right 0 rpm.

We applied 1000 rpm for all steps to ensure the reaction reaches the plateau quickly and consistently. Shaking slowly or turning shaking off typically slows down the reaction speed, as diffusion to the probe surface becomes rate limiting. Shaking ensures reagent exchange on the surface is due to binding kinetics.

Temperature control can also improve the stability and reproducibility of results. We advise setting the temperature at least 5°C above room temperature for stable use. In this example a temperature of 26°C improved signal stability, while room temperature and elevated temperatures up to 37°C were found less beneficial for immobilization (data not shown). The ideal run temperature may differ between molecules and assay protocol used.

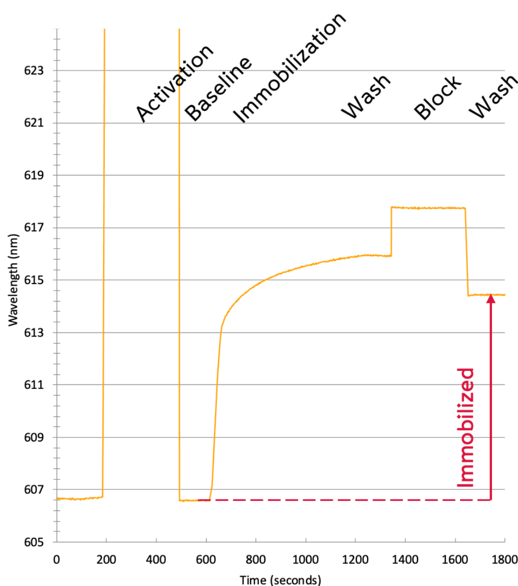


Figure 8: Example sensorgram of an optimized immobilization protocol.

After carrying out the optimization process for this example, the total time could be reduced to less than 30 minutes. In conditions of fast binding kinetics and further reduced reaction steps, the total time can decrease to 10 or even 5 minutes in total, while in conditions of slow binding kinetics you may prefer to keep the extended incubation and read times.

Discussion / Conclusion

Optimizing immobilization conditions is an important exercise to ensure reproducible results and good quality data from the quantification or binding experiment. This is due to differences between every capture molecule, and also each capture and target molecule pair.

Here we describe a pre-defined set of reaction conditions, supplemented with a stepwise approach to optimize the capture molecule immobilization on carboxyl FO-SPR probes. We use an example assay with an IgG class antibody bound as capture molecule for target molecule quantification.

In our experiments, a total immobilization shift of 8.48 nm with a CV as low as 2% was achieved. The result is a reproducible immobilization with high precision, reached with minimal consumable and reagent consumption during optimization. As target binding is your final goal, you may also opt to add a target binding step following this sequence to provide comparative data on the immobilization conditions. You can find more on this step in the white paper on label-free quantification.

With this procedure, you can use an easy approach to define and change run sequences on the White FOx software, without needing programming skills. Simply save and retrieve a protocol and edit the parameter or step you want to change, while leaving the rest of the sequence untouched.

Finally, this approach can also demonstrate the ease of use of probe dipping as a non-destructive sampling method. No waste of sample, and no issues with clogging or contaminating microfluidic channels, yet still obtaining true FO-SPR data in a fast and efficient way.

References

https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0017125_EDC_UG.pdf

Related white papers:
How to set up a label-free quantification assay starting from an immobilized capture surface is described in white paper 2 on label-free quantification.

Contact

info@foxbiosystems.com
+32 11 28 69 73

FOx BIOSYSTEMS NV
BioVille, Agoralaan Abis
3590 Diepenbeek, Belgium
www.foxbiosystems.com

WHITE FOx is for research use only,
not for diagnostic purposes.