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APPLICATION NOTE

Label-free protein quantification in bioproduction samples

Application note 4 | Version 2 | Kris Ver Donck[2], Dagmara Minczakiewicz[2], Kim Stevens[2], Gert Struys[1], Thomas Geuens[1], Koen Dierckx[1], Filip Delport[2]
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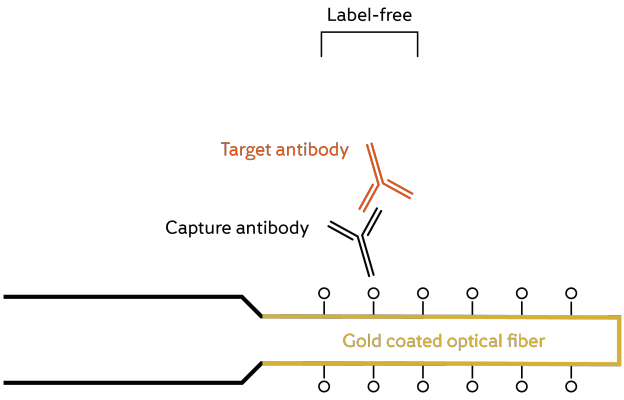
Abstract

This application note demonstrates the potential of FOx BIOSYSTEMS' fiber-optic surface plasmon resonance technology (FO-SPR) for label-free quantification of an antibody in cell culture media. We demonstrate the detection of a monoclonal IgG produced using a continuous perfusion platform. As an example system, we used the therapeutic monoclonal antibody, Trastuzumab (TRA), also known as Herceptin® which is used for the treatment of HER-2 positive tumors.

The methods described here demonstrate that FO-SPR can detect relevant concentrations of TRA in samples obtained from a bioreactor. TRA levels in the samples ranged from 5.8 to 139.1 µg/ml. A calibration curve from 0.31 to 12.5 µg/ml Herceptin® was constructed by making serial dilutions in blank cell culture media. There is an excellent correlation between results obtained by FO-SPR and those using an optimized ELISA technique (Spearman correlation up to 0.992).

We discuss here how the FO-SPR label-free assay can provide a fast and performant alternative to ELISA for the analysis of biomolecules in crude samples with minimal processing, thereby showing great potential as an R&D and analysis tool for bioproduction development.



Introduction

When optimizing or monitoring bioproduction runs, it is essential to accurately monitor the production of monoclonal antibodies (mAb). For this purpose, a fast and reliable method to determine protein concentration is required.

Crude biological samples often present challenges when quantifying biomolecules of interest during the research, development and bioproduction of diagnostics or therapeutics. To overcome these challenges, purification steps may be used, but these can introduce a bias in the experimental results and add more hands-on time and cost. Therefore, a direct method of detection is often preferred.

Here, we use Trastuzumab (TRA) as an example system to demonstrate direct detection and quantification in crude samples. TRA, also known as Herceptin® among other brand names, is a therapeutic monoclonal antibody used in the treatment of HER-2 receptor tumors, e.g. in breast carcinoma. This mAb is typically produced using a CHO cell line stably transduced with an optimized expression vector.

The most commonly used detection method for proteins is enzyme-linked immunosorbent assay (ELISA). However, this is a slow technique which takes around 1.5 days to give a result. A faster ELISA test has been developed for TRA detection in serum samples (1), but this still remains a laborious and time-consuming procedure. Furthermore, ELISA is typically run in batches, with samples collected over a period of time, making the time to result even longer. This delays the assessment and related decisions regarding the production batch. With White FOx you can run samples immediately after sample collection from the bioreactor, allowing fast and accurate monitoring of the bioreactor rather than retrospectively.

Surface plasmon resonance (SPR) is becoming increasingly used as a biosensing technology and offers a real-time alternative to ELISA that can be used with or without labels. SPR can measure local refractive index changes caused by the binding of suspended analytes and immobilized biomolecules on the sensor surface, resulting in a mass sensitive sensor. The most commonly used SPR biosensors are sensitive, reliable and precise. However, they typically use microfluidics and an optical path with prisms coated with a thin gold layer and are often unsuitable for quick testing due to their size, complexity and cost.

Fiber-optic surface plasmon resonance (FO-SPR) offers a convenient alternative to both conventional SPR biosensors and ELISA. Here, the gold layer is applied to fiber optic SPR probes which can be dipped into the sample of interest. This approach capitalizes on the speed and performance of SPR in an easy-to-use dip-in protocol, while eliminating the contamination and clogging issues often encountered when analyzing a series of crude samples with microfluidics instruments. FO-SPR is conveniently available in a benchtop instrument: the White FOx by FOx BIOSYSTEMS.

This application note describes the label-free quantification of Trastuzumab by FO-SPR in crude samples obtained from bioreactor sampling. Expected TRA levels in bioreactor samples are typically between 5 and 1,000 µg/ml. Direct detection in a label-free FO-SPR format is ideally suited to this application due to its speed, sensitivity, and ease of use. Its fluidics-free protocol requires minimal processing of crude samples. As sample concentrations are rather high, only dilution was required to match the dynamic range of the analysis. For even more sensitive analysis of proteins in crude samples with minimal sample processing, please see application note 2: Sensitive protein quantification in crude serum samples using a fast sandwich amplification assay.

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 as shown in figure 1. The sensor probe is simply dipped into the liquid sample to measure biomolecular interactions directly.

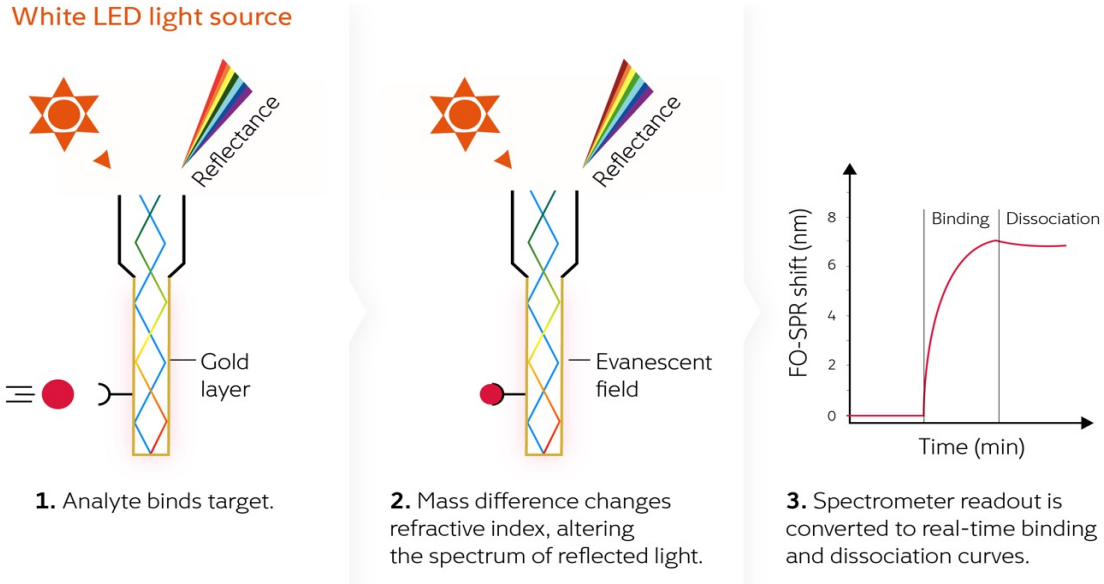


Figure 1: FO-SPR principle

Tools and reagents

To perform the procedures in this application 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
- On-desk refrigeration for protein solutions
- A microtube mixer and centrifuge

Buffer/Reagent	Concentration
MES: 2-(N-morpholino) ethanesulfonic acid, pH 6.0	50 mM
EDC: 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide	0.4 M in EDC/NHS
NHS: N-hydroxysuccinimide	0.1 M in EDC/NHS
Anti-IgG capture antibodies	7.5 µg/ml
PBS: Phosphate buffer saline, pH 7.4	10 mM
Ethanolamine in PBST (EtAm-HCl), pH 8,5	50 mM
Trastuzumab (Herceptin®)	1 mg/ml stock
Tween-20	0.01% in PBS

Table 1: Reagents and buffers. All solutions were prepared with deionized water purified by a Milli-Q Plus system.

Note: Special attention is required for EDC/NHS which is a highly reactive and short-lived reagent (EDC user guide, Sigma Aldrich) and should be prepared just prior to use. Separately dissolve NHS and EDC no more than 5 minutes before use, 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 as explained in the application note number 1 about immobilization on carboxyl probes. Thaw one aliquot of each at room temperature for about 20 minutes before use and mix in the designated wells just before placing the reagent in the instrument.

Generation of TRA production samples

simAbs NV has developed a unique benchtop scale production platform that allows for a continuous production of therapeutic monoclonal antibodies and has expertise in bioproduction, suspension CHO cell line generation, and cell culture media optimization. In order to accurately monitor bioproduction runs, a daily sampling of CHO suspension cells is performed via a built-in sample port in the bioreactor. To quantify the mAb concentration, samples are consequently centrifuged at 200xg for 5 minutes in order to obtain a good separation between cells and processed culture media. The obtained supernatant is then used for analysis using the White FOx.

Surface functionalization of FO probes with capture antibody

The methods in this application note build on the probe surface chemistry optimization for carboxyl FO-SPR probes as described in application note 1: Carboxyl probe immobilization for label-free protein quantification. As a different capture antibody was used here, the pH and concentration optimization experiments described in app note 1 were performed, and the optimal conditions specific to the TRA assay are described below.

Carboxyl probes were primed in MES buffer prior to activation. Carboxyl groups were activated by incubation in 0.4 M EDC/0.1 M NHS in MES buffer for 5 minutes. A 7.5 µg/ml solution of anti-TRA monoclonal capture antibody in a 10 mM MEST buffer at pH 6.0 was covalently immobilized to the activated carboxyl groups for 15 minutes. EtAm quenching was used to stop the capture antibody immobilization and a MES buffer wash removed non-covalently bound antibodies. All protocol run steps were performed as shown in table 2, with 1000 rpm shaking and sample temperature control set to 26ºC.

Step#	Step name	Position	Time (s)	Step function
1	Probe pick-up	A1		Probe signal reference
2	50 mM MES, pH 6.0	A2	600	Buffer equilibration
3	EDC/NHS	A1	300	Activation of carboxyl groups
4	10mM MES + 0.01% Tween-20 pH 5.7	A3	300	Buffer Baseline for immobilization
5	Capture antibody	A4	900	Covalent immobilization
6	50 mM EtAm-HCl pH 8.5	A5	300	Surface quenching
7	10 mM MES + 0.01% Tween-20 pH 5.7	A6	300	Wash
8	PBST (0.01% Tween), pH 7,2	A7	300	Target binding baseline *
9	Trastuzumab/Sample	A8	30	Target binding *
10	PBST (0.01% Tween-20), pH 7,2	A9	300	Wash
11	Probe drop off			

Table 2: Programmed run sequence in White FOx software. All steps are run with shaker 'on' at 1000 rpm and temperature control set to 26ºC. Total estimated runtime: 58 minutes.* Actual assay steps are step 8 and 9, giving an assay TTR of 5 minutes 30 seconds

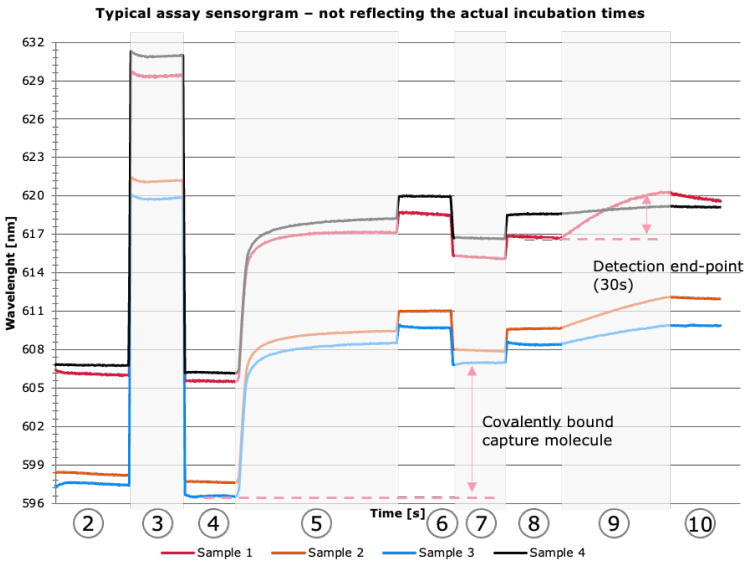


Figure 2: A typical FO-SPR sensorgram showing all steps of covalent immobilization of antibodies (5-7) and subsequent TRA detection (9) using the label-free bioassay approach as described in the run sequence of Table 2. Recordings shown represent 4 different concentrations of Trastuzumab applied in step (9).

Establishing the FO-SPR assay for TRA detection in crude cell media lysates

FO probes functionalized with anti-IgG capture antibodies were used to detect TRA diluted in PBS with 0.01% Tween 20 for 10 minutes as shown in Figure 2. Evaluation of the TRA binding curve and multiple time parameters of the TRA endpoint detection identified that an endpoint measurement after 30 seconds of TRA binding versus pre-binding baseline yielded the optimal balance between the dynamic range of the calibration curve and the time to result (TTR). For assay optimization, probes were first dipped in samples for 10 minutes to identify the optimal binding time. During subsequent routine testing setup, the functionalized FO probes were immersed in the TRA dilution for only 30 seconds and FO-SPR shift at 30 seconds versus baseline was calculated as the endpoint binding measurement.

To obtain a calibration curve, a concentration series of TRA (0.31, 0.63, 1.25, 2.5, 5.0, 7.5, 10.0, 12.5 µg/ml) was spiked into PBS / 0.01% Tween 20 buffer and run with the default protocol shown in Table 2. The 4PL model with weighting (1/y2) was used to calculate the calibration curve, as this was determined to provide a better fit at lower concentrations than the standard 4PL model. Verification of precision and accuracy was established by comparing the back calculation of the model concentrations from the measured SPR shifts to the input concentrations.

TRA quantification in samples using FO-SPR

TRA concentration was determined from 7 de-identified crude samples, 5 spiked samples, and 2 blanks, using the methods described in the above section. Samples and blanks were diluted 50-fold, 100-fold, or 200-fold in assay buffer to fit within the calibration curve range and measured in quadruplicate.

TRA quantification in samples using ELISA

A total human IgG ELISA kit (Thermo Fisher Scientific, Invitrogen, Cat 88-50550) was used to determine IgG concentrations in clarified harvest. The ELISA plate was first coated overnight with an anti-IgG human monoclonal antibody, then blocked and washed with PBS-Tween (0.05%). Samples, blanks and standards were applied, followed by additional washing steps. An HRP-conjugated anti-human monoclonal secondary detection antibody was then applied, using TMB as substrate. The reaction was stopped using 1M phosphoric acid. The detailed protocol description can be found online on the product information sheet.

A standard curve ranging from 100ng/mL to 1.56ng/mL was established. Samples were diluted as required, depending on the expected concentration of collected samples (expected TRA concentrations between 0.02-1mg/mL). Samples were centrifuged (5min; 200xg) before applying them to the plate, to reduce potential matrix signals caused by the harvest environment (cells, host cell proteins, etc). Samples were diluted 4,000-fold, 8,000-fold and 16,000-fold based on an expected concentration of 0.2-0.5mg/mL. Sample concentrations were back calculated based on dilution factors and interpolation of raw sample absorbance data (AU), using the linear regression function of the standard curve. Every measurement was performed at 450nm in duplicate. The acceptable %CV for duplicate measurements was set at 20%.

Results

Non-specific binding

TRA only binds specifically to the biosensor in the presence of capture antibody. The interaction between the cell culture media and immobilized capture antibody was low. The capture antibodies have a high specificity against IgG (1). The largest signal-to-noise ratio and smallest inter-experiment variability was achieved using ethanolamine in PBST. This was therefore used as quenching buffer in all subsequent experiments.

Calibration curve generation

In order to quantify TRA in crude lysate samples, calibration curves were generated for TRA spiked in buffer-diluted cell culture medium, where the SPR shifts were plotted against a function of concentration, as shown in Figure 3.

The concentrations of unknown samples were checked by interpolating the SPR signal into the calibration curve. Less than 10% difference was observed between known and determined concentrations from interpolation with the calibration curve.

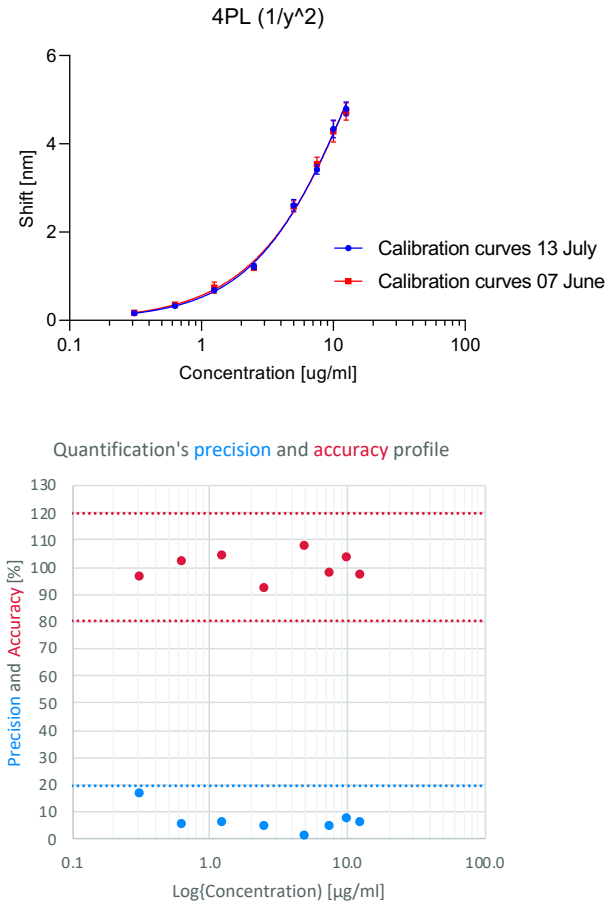


Figure 3:Top: Calibration curve for spiked TRA in buffer-lysate. Error bars represent standard deviation. Bottom: precision and accuracy profile plot for the calibrator samples.

As the concentration curve for binding assays is typically not linear, it is possible to use multiple models in the analysis software to identify the best curve fitting model to run concentration curves.

Typically, the higher order models give superior fitting results for complex non-linear data, but this comes at the price of analyzing enough meaningful datapoints in the curve. In Table 3 shows a list of common fitting models, together with the minimum required number of datapoints. For calibration curves, the higher order models are commonly used because they provide superior matching to the actual data. However, the number of independent data points required increases with the complexity of the model applied. Further optimizations are possible by using transformations on the input data, such as the 1/y2 used in this study.

Model	Minimum # datapoints	comment
Interpolation	5	Very crude model, requires multiple replicates per datapoint, limited validity
Linear	3	Approximation based on assumption of linearity, typically not suited as calibration curve binding assays
2nd order polynomial	4	Parabolic approximation, typically not suited as calibration curve for binding assays
3rd order polynomial	5	Basic calibration curve approximation for binding assays
4PL	6	Common calibration curve approximation for binding assays
5PL	7	Complex calibration curve approximation for binding assays

Table 3: Common standard curve fitting models.

TRA measurement in unknown samples

For FO-SPR, both the coefficient of variation between the four measurements of each individual sample and the inter-assay coefficient of variation were less than 10%.

There was excellent correlation between TRA concentrations measured with FO-SPR and ELISA, as shown in Figure 4 (Pearson correlation coefficient: 0.992 at 50-fold dilution and 0.928 at 100-fold dilution).

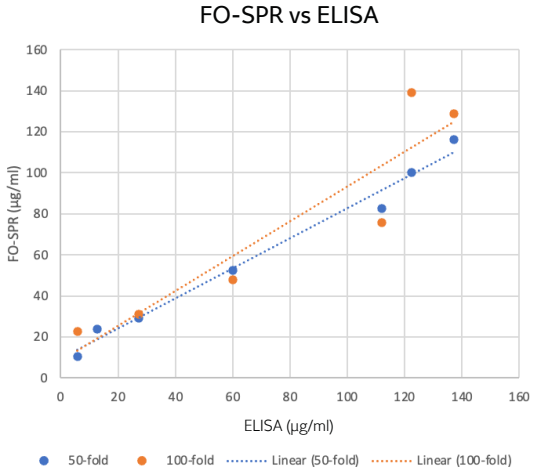


Figure 4: Evaluation of the FO-SPR assay with crude bioreactor samples compared to the results from the anti-IgG ELISA test. Data plotted as undiluted initial sample concentrations. Pearson correlation coefficient: 0.992 at 50-fold dilution and 0.928 at 100-fold dilution.

Discussion / Conclusion

When measuring antibodies in research samples, it is essential for tests to be sufficiently sensitive to detect and quantify at relevant concentrations. In addition, the ability to deal with complex crude samples, such as samples from cell culture media, without the need for time-consuming purification procedures to avoid cross-contamination or clogging in microfluidics systems is highly advantageous.

TRA concentrations in these bioproduction samples can typically range from 5 to 1,000 µg/ml, depending on the culture phase. With a lower limit of the calibration curve of 0.31 µg/ml, FO-SPR label-free assays show great potential for this application.

Applying 50- and 100-fold dilutions matched the respective samples to the dynamic range of the calibration curve. The results obtained by FO-SPR showed excellent correlation with those obtained from an ELISA test, the current reference standard for TRA quantification.

In addition, inter-assay coefficient of variation was less than 10% when testing patient samples with FO-SPR and there was very little non-specific binding between the various assay components (antibodies, target molecule and sensor surface). Furthermore, the fluidics-free setup means that crude samples can be analyzed with minimal processing without the risk of clogging or contamination associated with microfluidics-based SPR systems.

As presented here, the total assay time of just under 1 hour including capture antibody immobilization is already substantially faster than the ELISA assay, which typically has an overnight incubation. With pre-functionalized probes stored in assay buffer, the assay time can be further reduced to less than 6 minutes.

Furthermore, since FO-SPR is fully automated and can either run individual measurements or with 4 parallel FO sensors, it shows great potential to quickly determine antibodies produced in samples from cell culture bioreactors, both for a limited number of samples at the time, as well as in high throughput mode.

References

1. B. Cardinali et al. (2014) Trastuzumab quantification in serum: a new, rapid, robust ELISA assay based on a mimetic peptide that specifically recognizes trastuzumab. Anal Bioanal Chem. 406 (18), 4557-61; DOI: 10.1007/s00216-014-7842-4

Related application notes

Application note 1: Carboxyl probe immobilization for label-free protein quantification, describes how to optimize immobilization to the carboxyl FO-SPR probes.
Application note 2: Sensitive protein quantification in crude serum samples: comparison of FO-SPR and ELISA, describes a sensitive sandwich FO-SPR protocol for quantifying proteins in complex matrices.
Application note 3: Sensitive protein quantification in blood, dried blood spots, serum and plasma, describes a ten-minute FO-SPR protocol for quantifying proteins in complex matrices, including whole blood.

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