Journal of Chromatography B, 878 (2010) 264–270

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Label-free and amplified quantitation of proteins in complex mixtures using diffractive optics technology☆

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ARTICLE INFO

Article history:
Received 31 March 2009
Accepted 17 June 2009
Available online 23 June 2009

Keywords:
Label-free
Biosensor
Diffraction
Protein A
Regeneration

ABSTRACT

Immunoaffinity approaches remain invaluable tools for characterization and quantitation of biopolymers. Their application in separation science is often limited due to the challenges of immunoassay development. Typical end-point immunoassays require time consuming and labor-intensive approaches for optimization. Real-time label-free analysis using diffractive optics technology (dot®) helps guide a very effective iterative process for rapid immunoassay development. Both label-free and amplified approaches can be used throughout feasibility testing and ultimately in the final assay, providing a robust platform for biopolymer analysis over a very broad dynamic range. We demonstrate the use of dot in rapidly developing assays for quantitating (1) human IgG in complex media, (2) a fusion protein in production media and (3) protein A contamination in purified immunoglobulin preparations.

1. Introduction

1.1. Overview of diffractive optics technology (dot®)

dot combines grating-based light diffraction and immobilized affinity surfaces to produce a sensitive and accessible platform for the detection of biomolecular interactions without the use of fluorescent or chemiluminescent labels. Specifically, capture molecules are immobilized on a flat surface in an optimized grating that produces a strong diffraction pattern when illuminated: when coherent light strikes a non-random pattern of capture molecules on the dot® Sensor, this results in constructive and destructive interferences which in turn produce a well defined diffraction image [1] (Fig. 1). Binding of biomolecules to the patterned capture molecules improves the diffraction efficiency of the pattern, which increases the diffracted signal intensity while the release of the interacting species leads to a measurable change in signal. Diffraction sensing is inherently more robust than other optical sensing methods because it is self-referencing [2]. With dot, the transduction of binding events is dependent on the initial pattern and an increase in diffractive signal intensity will only occur if molecules bind exclusively to the patterned capture reagents. In simplistic terms, the diffraction efficiency depends on the relative height difference between the patterned and non-patterned areas, thus non-specific binding to both the patterned and non-patterned regions will not affect the signal significantly (Fig. 2). This attribute provides a considerable advantage over other optical biosensor systems in which any surface binding event will cause a spurious increase in signal [2,3].

Each sensor is manufactured with up to eight assay features along a linear flow channel (Fig. 3). The dotLab® System introduces samples and assay reagents into the dot Sensor using an automated sampling system and a high-precision fluidic controller: the operator can select one of several incubation modes including constant flow and a mixing mode for samples of limited volume. Fluidic procedures do not significantly affect the signal, reflecting the inherent self-referencing capabilities of diffraction. Table 1 shows a screen capture of the typical fluidic program: flow rates can range from 13 μL/min to 4000 μL/min and the user can choose to define contact time with the sample through cycle times or a defined period. Note that reagents are separated by an air gap which allows for proper separation of reagents. In addition, the introduction of air into the sensor leads to a transient spike in signal as well as a timing reference for each step and reagent addition (see Fig. 4).

Data acquisition for each feature can be specified by the user ranging in frequency from 0.1 to 10 Hz: the instrument will rapidly sweep over the selected features at the desired rate allowing for contemporaneous acquisition of data. Binding of target molecules to the patterned assay features is detected by interrogation with focused laser light in a total internal reflection mode. The detection beam never passes through the flow channel (see Fig. 3) [1], providing an ideal platform for assays on complex biological

☆ This paper is part of the special issue "Immunoaffinity Techniques in Analysis", T.M. Phillips (Guest Editor).

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doi:10.1016/j.jchromb.2009.06.027

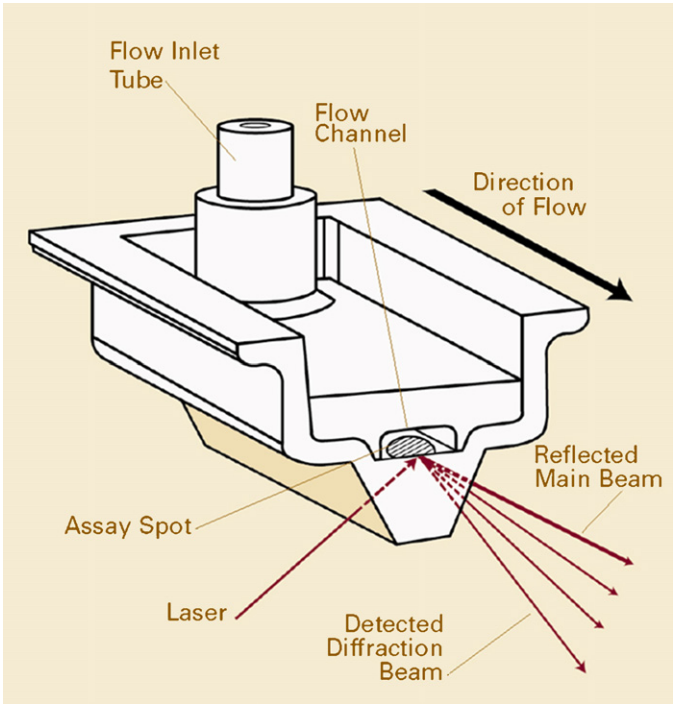


Fig. 1. Principles of diffraction-based sensing on the dotLab® System. The sample is interrogated in total internal reflection allowing for real-time interaction detection in complex media. The incident laser interacts with the assay spot or feature and is diffracted as a result of the diffraction grating efficiency which is linked to its relative height with respect to the sensor surface.

samples. We demonstrate the use of both label-free and amplified assays on the dotLab System to rapidly develop and execute quantitation methods for unpurified/purified proteins and their contaminants.

2. Materials and methods

2.1. Instrumentation—dotLab System (Axela Inc., Toronto, Ontario, Canada)

The dotLab System introduces samples and assay reagents into the dot Sensor using an automated sampling system and a high-precision fluidic controller: the operator can select one of several incubation modes including constant flow and a mixing mode for samples of limited volume. Assays are user defined combinations of fluidic steps that essentially mimic typical assay steps.

"Wash/Condition" steps are used to (1) prime the sensor at the beginning of an assay or (2) wash the sensor in between assay steps to flush out unbound reagent in the sensor and/or excess reagent in the tubing lines. "Load" steps are used to deliver samples and low-volume reagents from the sample tray into the sensor. "Tip Wash" steps perform an internal and external wash of the autosampler probe tips. It also serves to remove any hanging droplets of fluid at the end of the probes, preventing cross-contamination in sample tray locations. "Load Sample" steps allow loading of multiple samples with the same assay parameters in one method. It includes a looping feature that enables the user to assign a section of the method to repeat for the desired number of samples.

Note that operators can set several parameters, these are indicated in Tables 1–3 or choose from preexisting methods with pre-set parameters.

2.2. Sensors—dot-Avidin were used according to manufacturer's recommendations (Axela Inc., Toronto, Ontario, Canada)

The dot-Avidin can be used to immobilize a variety of biotinylated capture species, including antibodies, proteins, and oligonucleotides. Sensors are manufactured with a row of eight circular assay spots positioned along a linear flow channel. Each assay spot has a diameter of 2 mm, and is produced by immo-

Table 1
Method summary for full length IgG quantitation assay.

Step number	Reagent name	Reagent volume (μL)	Reagent flowrate (μL/min)	Air gap (μL)	Incubation type	Incubation volume (μL)	Incubation duration hh:mm:ss	Incubation flowrate (μL/min)
1: Wash/condition	HBST	2000	2000	-	Mix	30	00:00:30	2000
2: Tip wash	HBST	500	2000	-	-	-	-	-
3: Load	BSA	50	2000	5	Mix	10	00:03:00	500
4: Wash/condition	HBST	2000	2000	-	Mix	30	00:00:30	2000
5: Tip wash	HBST	500	2000	-	-	-	-	-
6: Load	Bt-ProtA	50	2000	5	Mix	10	00:10:00	500
7: Wash/condition	HBST	1000	2000	-	Mix	30	00:00:30	2000
8: Tip wash	HBST	500	2000	-	-	-	-	-
9: Load	HIgG 400ug	50	2000	5	Static	-	00:05:00	-
10: Wash/condition	HBST	1000	2000	-	Mix	30	00:00:30	2000
11: Tip wash	HBST	500	2000	-	-	-	-	-
12: Wash/condition	Regen	1000	2000	-	Mix	30	00:01:00	2000
13: Wash/condition	HBST	1000	2000	-	Mix	30	00:00:30	2000
14: Tip wash	HBST	500	2000	-	-	-	-	-
15: Load	HIgG 400ug	50	2000	5	Static	-	00:05:00	-
16: Wash/condition	HBST	1000	2000	-	Mix	30	00:00:30	2000
17: Tip wash	HBST	500	2000	-	-	-	-	-
18: Wash/condition	Regen	1000	2000	-	Mix	30	00:01:00	2000
19: Wash/condition	HBST	1000	2000	-	Mix	30	00:00:30	2000
20: Tip wash	HBST	500	2000	-	-	-	-	-
21: Load	HIgG xug	50	2000	5	Static	-	00:05:00	-
22: Wash/condition	HBST	1000	2000	-	Mix	30	00:00:30	2000
22: Tip wash	HBST	500	2000	-	-	-	-	-
24: Wash/condition	Regen	1000	2000	-	Mix	30	00:01:00	2000
25: Wash/condition	HBST	1000	2000	-	Mix	30	00:00:30	2000
26: Tip wash	HBST	500	2000	-	-	-	-	-
27: Load	HIgG xug	50	2000	5	Static	-	00:05:00	-

loop[a]

[a]Loop: After introduction of each subsequent sample or calibrator is subjected to the same 5 steps (Steps 22–26) prior to introduction of a new sample.

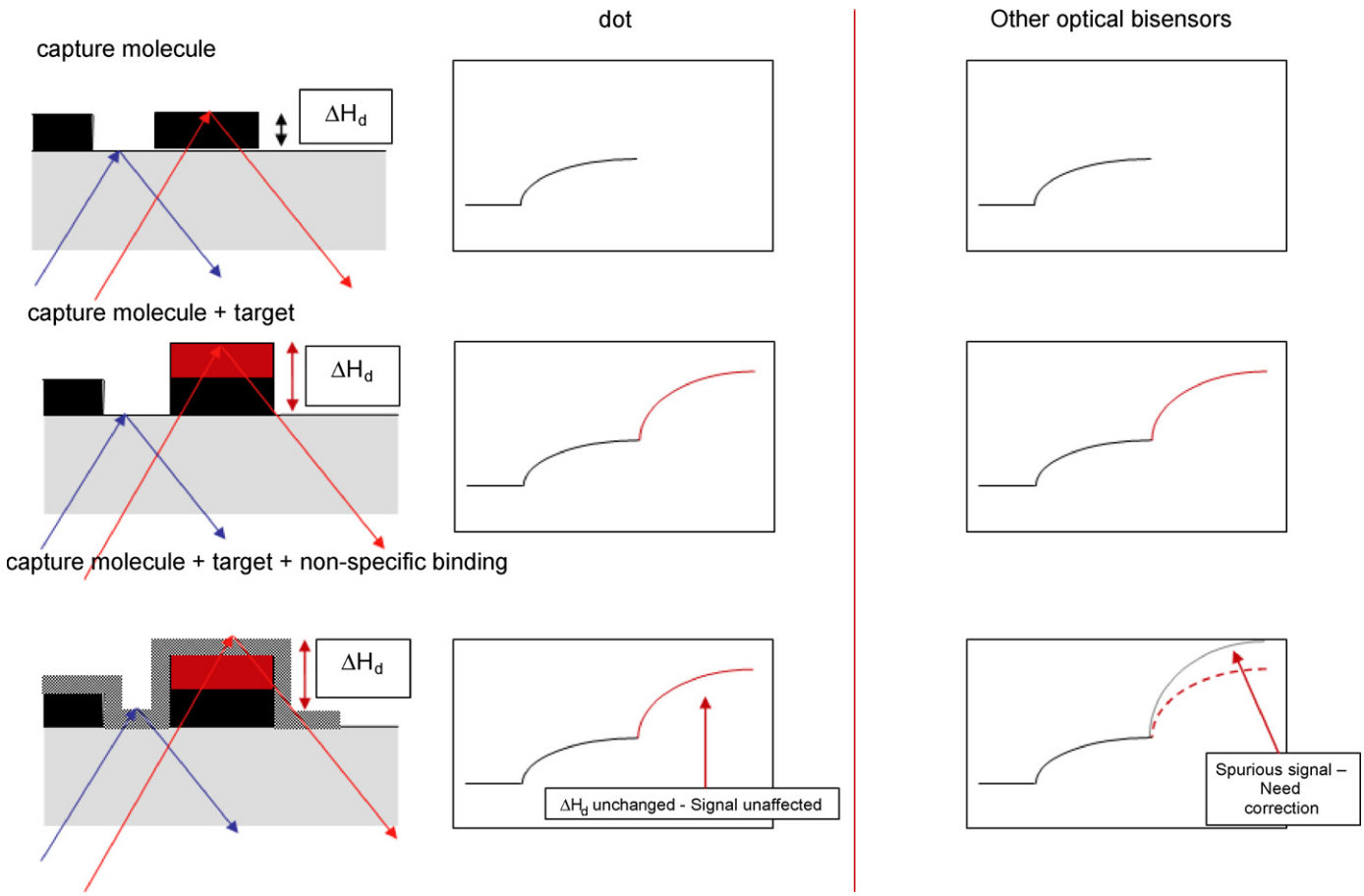


Fig. 2. Diffraction is inherently self-referencing.
Binding events and responses are represented schematically for dot and other label-free technologies. Non-specific binding has little or no effect on dot signal as it does not impact the differential height (hence diffraction efficiency) of the grating while it causes spurious signal on other optical platforms which require compensation using reference channels.

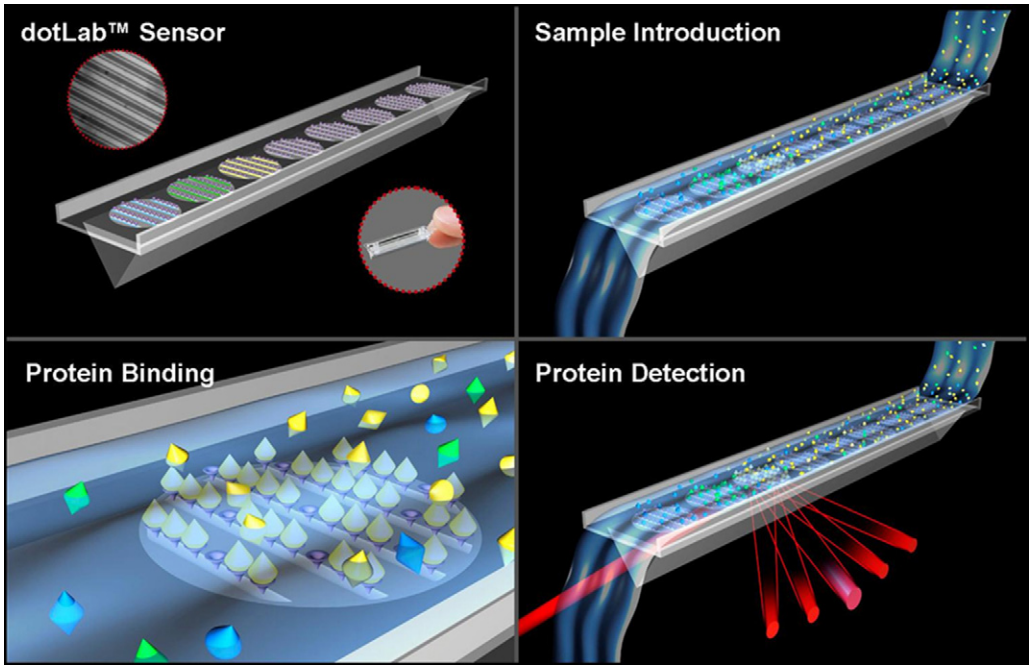


Fig. 3. Detecting biomolecular interactions in real-time with the dotLab System and dot Sensor. Up to 3 features can be monitored on the avidin sensor for replicate determinations. Future offerings from Axela will allow for up to 8 features to be monitored simultaneously.

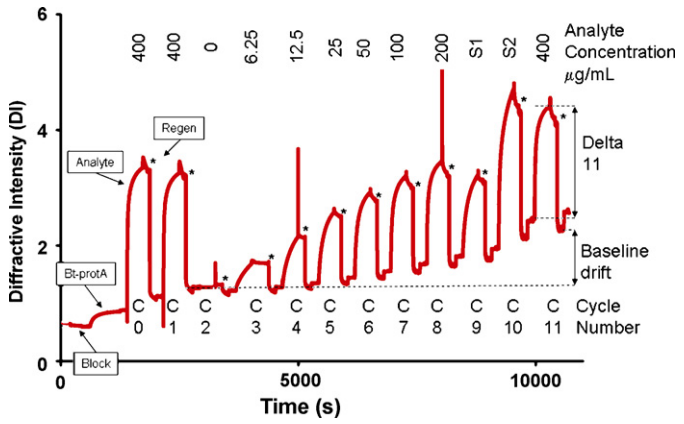


Fig. 4. Representative trace from a regeneration based quantitative assay for full length human IgG.
Following immobilization of biotinylated protein A, IgG calibrators and test samples are sequentially applied to the sensor; after each sample addition, the sensor is washed with a regeneration solution (Regen) to remove bound IgG molecules but leaving the biotinylated protein A in place. The increase in diffractive intensity can be seen to be proportional to the concentration of IgG added. Each cycle of regeneration is highlighted by a *. Baseline drift from the initial surface level until the end of the protocol is indicated.

bilizing avidin capture molecules in a diffractive grating pattern. Each avidin assay spot has an estimated biotin binding capacity of approximately 15 fmol. The flow channel's depth is 0.25 mm; the total volume of the sensor's flow channel is 10 μL. Each side of the flow channel is connected to a length of soft polyvinyl chloride tubing.

2.3. Reagents and samples

TMB 1-component™ Peroxidase Substrate, a precipitating form of 3,3′,5,5′-tetramethylbenzidine (TMB), was obtained from KPL Inc. (Gaithersburg, MD). Running buffer consisting of PBS (0.154 M NaCl, 0.01 M phosphate) with 0.025% Tween-20 (v/v), pH 7.4; bovine serum albumin (BSA) blocking buffer (5 mg/mL of BSA in running buffer), HBS buffer (0.15 M NaCl, 0.01 M HEPES-Na, pH 7.3), 50 mM NaOH, 10 mM glycine, pH 3.0, concentrated Tween 20 (10%, w/v in H_2O), biotin protein A, capture and detector antibodies for protein A residual assay were from Axela. HCl was from Sigma–Aldrich (UK). Samples from cell culture supernatants and purified protein preparations were kindly provided by a collaborator. Calibrators for full length IgG or fusion protein were made by diluting concentrated stocks (provided by collaborator) in the appropriate cell culture supernatant matrix.

Table 2
Method summary for Fc-fusion protein quantitation assay.

Step number	Reagent name	Reagent volume (μL)	Reagent flowRate (uL/min)	Air gap (μL)	Incubation type	Incubation volume (μL)	Incubation duration hh:mm:ss	Incubation flowrate (μL/min)
1: Wash/condition	HBST	2000	2000	-	Mix	30	00:00:30	2000
2: Tip wash	HBST	500	2000	-	-	-	-	-
3: Load	BSA	50	2000	5	Mix	10	00:03:00	500
4: Wash/condition	HBST	2000	2000	-	Mix	30	00:00:30	2000
5: Tip wash	HBST	500	2000	-	-	-	-	-
6: Load	Bt-ProtA	50	2000	5	Mix	10	00:10:00	500
7: Wash/condition	HBST	1000	2000	-	Mix	30	00:00:30	2000
8: Tip wash	HBST	500	2000	-	-	-	-	-
9: Load	1mg Fusion	50	2000	5	Mix	10	00:05:00	500
10: Wash/condition	HBST	1000	2000	-	Mix	30	00:00:30	2000
11: Tip wash	HBST	500	2000	-	-	-	-	-
12: Wash/condition	Regen	1000	2000	-	Mix	30	00:01:00	2000
13: Wash/condition	HBST	1000	2000	-	Mix	30	00:00:30	2000
14: Tip wash	HBST	500	2000	-	-	-	-	-
15: Load	1mg Fusion	50	2000	5	Mix	10	00:05:00	500
16: Wash/condition	HBST	1000	2000	-	Mix	30	00:00:30	2000
17: Tip wash	HBST	500	2000	-	-	-	-	-
18: Wash/condition	Regen	1000	2000	-	Mix	30	00:01:00	2000
19: Wash/condition	HBST	1000	2000	-	Mix	30	00:00:30	2000
20: Tip wash	HBST	500	2000	-	-	-	-	-
21: Load	Xug Fusion	50	2000	5	Mix	10	00:05:00	500

loop[a]

[a]Loop: After introduction of each subsequent sample or calibrator is subjected to the same 5 steps (Steps 16–20) prior to introduction of a new sample.

Table 3
Method summary for protein A quantitation assay.

Step number	Reagent name	Reagent volume (μL)	Reagent flowrate (μL/min)	Air gap (μL)	Incubation type	Incubation volume (μL)	Incubation duration hh:mm:ss	Incubation flowrate (μL/min)
1: Wash/condition	PBST	1000	4000	–	Mix	50	00:00:30	4000
2: Tip wash	PBST	1000	4000	–	–	–	–	–
3: Load	BSA	50	2000	5	Mix	10	00:03:00	2000
4: Load	Bt-capture	50	2000	0	Mix	10	00:10:00	2000
5: Wash/condition	PBST	1000	2000	–	Mix	50	00:00:30	2000
6: Wash/condition	PBST	1000	2000	–	Mix	50	00:00:30	2000
7: Wash/condition	PBST	1000	2000	–	Mix	50	00:00:30	2000
8: Wash/condition	PBST	1000	2000	–	Mix	50	00:00:30	2000
9: Tip wash	PBST	1000	2000	–	–	–	–	–
10: Load	TMB	50	2000	5	Static	–	00:05:00	–
11: Wash/condition	PBST	1000	4000	–	Mix	50	00:00:30	4000
12: Tip wash	PBST	1000	4000	–	–	–	–	–

2.4. Fluidic methods

The fluidic methods are summarized in Tables 1–3.

2.5. Protein A assay protocol

Briefly, the assay employs a simple immunosandwich with a biotinylated capture antibody and a detector antibody coupled to Horseradish Peroxidase (HRP) (Axela Inc., Toronto, Ontario, Canada). Both are specific to protein A. Samples are treated at 95 °C and allowed to cool to room temperature. The samples are spun at 14,000 rpm to remove any precipitating material. The samples are then combined with both the capture and detector antibodies and incubated at room temperature for at least 30 min. The final concentration of each component in this mixture was as follows: the biotinylated capture antibody is present at 1 μg/mL, the detector antibody coupled to HRP is present at 3 μg/mL, the human IgG (huIgG) concentration was 2.5 mg/mL in a PBS matrix containing BSA at 5 mg/mL supplemented with 0.1% gelatin, 150 mM NaCl and 0.05% Tween 20. The biotinylated capture antibody–protein A–detector antibody complex is immobilized on the avidin sensor through the biotin molecules on the capture antibody. Following the capture of the complex on the avidin sensor, TMB is introduced and used to detect the low abundance protein A residual. Every component is introduced through a precise fluidic method and defined incubation time, see Table 3. The procedure is carried out on an individual sensor for every sample and calibrator in the appropriate matrix. A series of calibrators were produced using 2-fold serial dilutions from 1 ng/mL and 0.016 ng/mL protein A in the presence of 2.5 mg/mL huIgG.

2.6. Data analysis

All data were exported as comma separated values (csv) files and analyzed using the Data Analysis Manager (Axela Inc., Toronto, Ontario, Canada) for visualization and overlays and GraphPad Prism® (GraphPad Software Inc., San Diego, CA) for normalization and Boltzmann-Sigmoidal fits.

3. Results and discussion

3.1. Rapid quantitation of full length human IgG—assay development

In order to rapidly devise a method to quantitate full length human IgG in production media, a regeneration-based protocol was scouted. In this regeneration based protocol, the capture agent, a biotinylated protein A is immobilized on the surface and cycled through a loop of sample introduction/measurement and wash in a regeneration solution (abbreviated regen in Table 1) which strips away the bound analyte and allows recovery of the capture reagent surface such that it is available for quantitation of the next sample. With this approach, the total assay time for the generation of the calibration curve and test samples was 2 h. We assayed three separate regeneration protocols involving (1) 0.1 M HCl, (2) 50 mM NaOH and (3) 10 mM glycine, pH 3.0. Glycine proved to be the better choice for this particular combination of capture agent, a biotinylated protein A, and the analyte of interest in its matrix, a full length human IgG in tissue culture supernatant. The other approaches led to a very significant updrift in the signal baseline or did not allow for satisfactory recovery of a specific calibrator signal over the 11 cycles needed for the experiment (data not shown). The efficacy of the regeneration protocol was primarily assessed by comparing the signal generated at cycles 0, 1 and 11 when a 400 μg/mL calibrator is introduced (see Fig. 4): in this particular example the signal generated at cycle 11 was over 90% that of cycle 1, indicated as Delta 11 in the figure. Here, the avidin is first blocked with a BSA containing solution and then incubated with the biotinylated protein A to immobilize it on the surface. Following a few quick wash steps, sample is introduced and a binding event at the capture surface is detected as an increase in diffractive intensity (DI): the increase over the baseline is measured as a delta. Sample incubations were performed in a static mode so that binding would be diffusion limited and concentration dependent. At these high concentrations, mixing or continuous flow modes would likely give rise to very rapid saturation that would be intractable for calibration and quantitation. We have normalized and overlaid the binding curves for each cycle in Fig. 5 for evaluation. The real-time observation of binding allows us the flexibility to select either a rate based (initial) or time-point derived calibration curve. In this example, we selected a 10 s time-point based calibration curve to generate the curve seen in Fig. 6. Two test samples were determined to have 52.1 μg/mL and 794 μg/mL once we corrected for the dilution of the sample. Similar concentration values were independently obtained for these samples using different quantitation methods (data not shown). Therefore, with a simple experimental approach we were able to provide satisfactory quantitation of target analyte in production media. The flexibility in assay design and the ability to

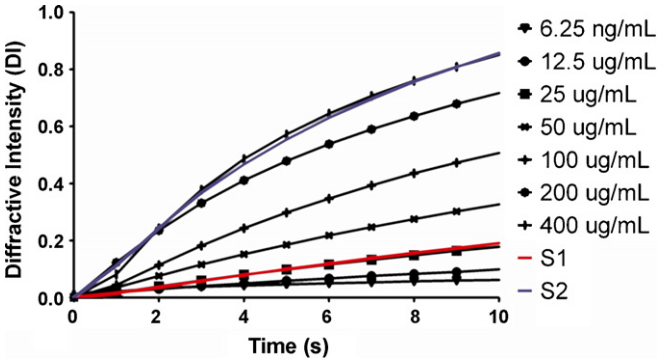
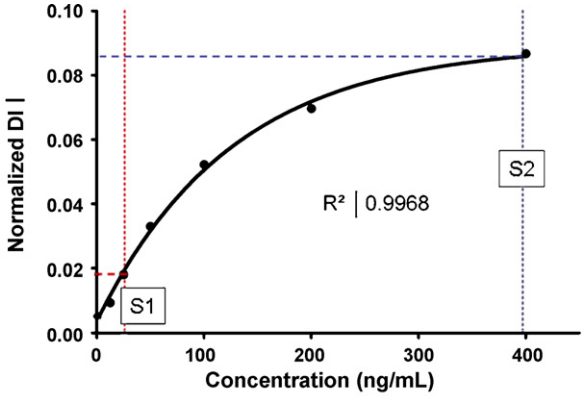


Fig. 5. Overlay of normalized analyte binding zones from cycles 2–11.
Analyte binding zones from each cycle were normalized to the origin to facilitate comparison. Binding responses are proportional to analyte concentrations. A time point (t = 10 s) providing reproducible discrimination for each calibrator concentration was selected.



Sample	Derived Value	Dilution Factor	Concentration
S1	26.06 μg/mL	2	52.1 μg/mL
S2	397 μg/mL	2	794 μg/mL

Fig. 6. Calibration curve for full length human IgG quantitation.
Normalized diffractive intensities for each calibrator concentration at the 10 s time point were plotted against their respective concentration. A Boltzmann-Sigmoidal fit was used for the curve. R^2 value for the fit was 0.997. The determinations for the 2 unknown samples S1, S2 are indicated on the graph.

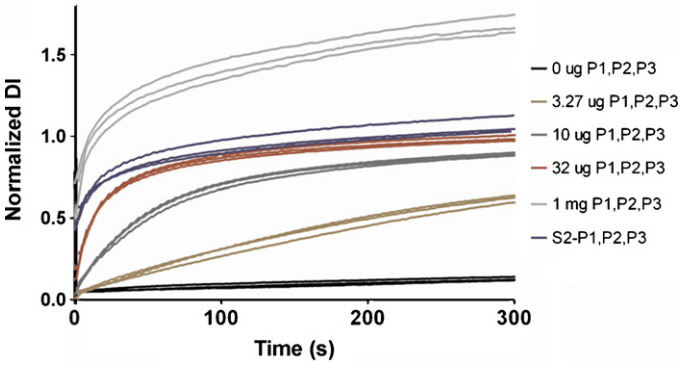


Fig. 7. Overlay of representative sample of normalized analyte binding zones for quantitation of 46 kDa fusion protein.
Analyte binding zones from representative cycles were normalized to the origin to facilitate comparison (zeroed DI on *y*-axis). Binding responses are proportional to analyte concentrations. Multiple curves are shown for each sample representing three feature monitoring by the dotLab® System, each feature is indicated by P1, P2 or P3. A time point ($t = 5$ s) providing reproducible discrimination for each calibrator concentration was selected.

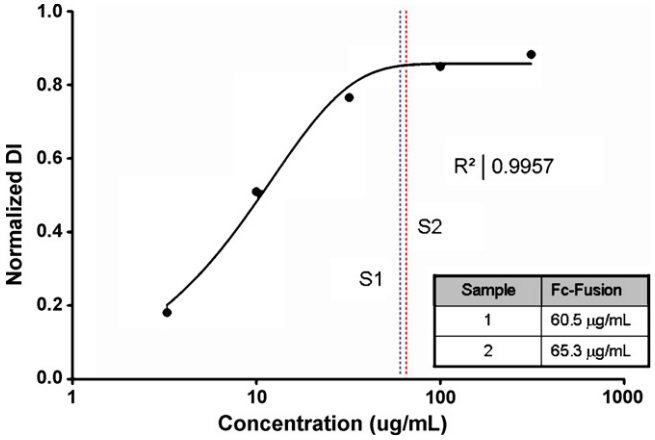


Fig. 8. Calibration curve for 46 kDa Fc fusion protein quantitation.
Normalized diffractive intensities for each calibrator concentration at the 5 s time point were plotted against their respective concentration. A Boltzmann-Sigmoidal fit was used for the curve. R^2 value for the fit was 0.996. The determinations for the 2 unknown samples S1, S2 are indicated on the graph.

observe the reactions in real-time allowed for rapid development of a quantitative assay.

3.2. Quantitation of fusion protein—multiplexed monitoring

The dotLab System allows the user to monitor up to 3 features on the dot-Avidin sensor. We built upon the previous method to quantitate a human Fc-fusion protein in production media. The method chosen was also based on regeneration cycles but 0.1 M HCl proved more effective in terms of regeneration (data not shown). In addition, we used a mixing mode for the sample/calibrator incubations (see Table 2). As described in the previous section, we normalized the data and evaluated the binding curves for each of the calibrator concentrations and the test samples. The raw aligned data is shown in Fig. 7. The actual coefficient of variation (CV) for each triplicate determination of the calibrators was less than 4%, reflecting the robust performance of the avidin sensors. It should be noted that this data can be further treated or fitted to improve the reproducibility. In previous experiments, with other model systems Axela has demonstrated that fitting can further reduce CV's to less than 1.0% (unpublished data). After normalization for baseline variations, five (5) second time point diffractive intensities were plotted against the analyte concentration to generate the calibration curve seen in Fig. 8. Our data indicated that samples 1 and 2 contained respectively 60.5 μg/mL and 65.3 μg/mL of fusion protein. This data correlates very well with the determinations obtained by an SPR based analysis, i.e. 60 μg/mL and 62.3 μg/mL. The total assay time including test samples and calibrators was 2 h. The triplicate determination facilitated by three feature monitoring provides a simple means to assess reliability and reproducibility of a particular assay set-up. Moreover, it can allow for identification of any spurious results and provides a method to rigorously exclude outliers.

3.3. Protein A residual assay

The low-cost sensor can not only be used in regeneration based protocols but also used in a disposable manner to minimize any risk of cross-contamination between samples. Furthermore, we can detect very low concentration analytes by using enzymatic amplification whereas use of such approaches on conventional optical biosensors would be intractable or too costly. In the following assay, we use an HRP coupled detector antibody to detect residual protein A in purified protein preparations.

A representative trace is shown in Fig. 9: after a brief wash and blocking step, the preincubated solution containing the test sample or calibrator as well as the biotinylated and HRP coupled detector antibodies is introduced onto the sensor. The small binding observed reflects the binding of both free biotinylated capture antibody and full immunocomplexes. In order to detect and quantitate the low abundance immunocomplexes, we introduced TMB which is converted by the HRP into a precipitating substrate. The precipitating substrate builds increased height on the diffraction grating, improving its diffraction efficiency and gives rise to an increase in diffractive intensity ([4]; see Fig. 9). The increase is proportional to the amount of capture immunocomplexes; moreover a calibration curve can be derived in this manner. We have overlaid the normalized traces from the TMB precipitation portion of the assays in Fig. 10 for ease of comparison. After examining the data, we determined that the optimal method for calibration would be a slope based determination, essentially an initial rate, after 10 s. The real-time observations helped us determine the kinetics of signal evolution for this TMB and HRP combination, something that is nearly impossible with classic ELISA's [7]. Even

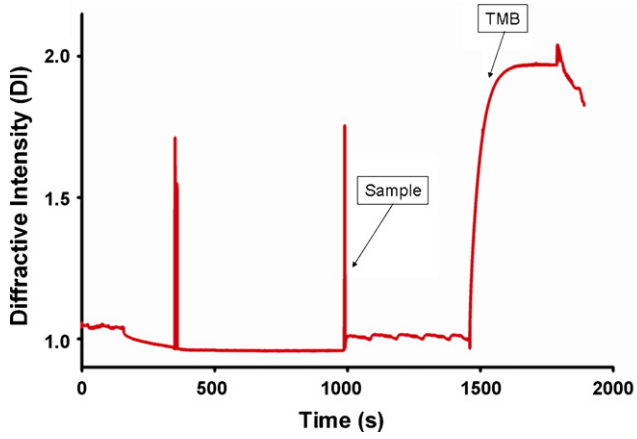


Fig. 9. Representative trace for a quantitative assay for residual protein A.
The sensor surface was first washed and blocked, and the preincubated protein A sample containing biotinylated and HRP anti-protein A antibodies was added. Following sample incubation, a further wash was performed and the precipitating substrate TMB introduced. A large diffractive intensity change is observed as the HRP-containing complex on the sensor surface cases the TMB to precipitate, building height on the diffractive grating and resulting in increased diffraction.

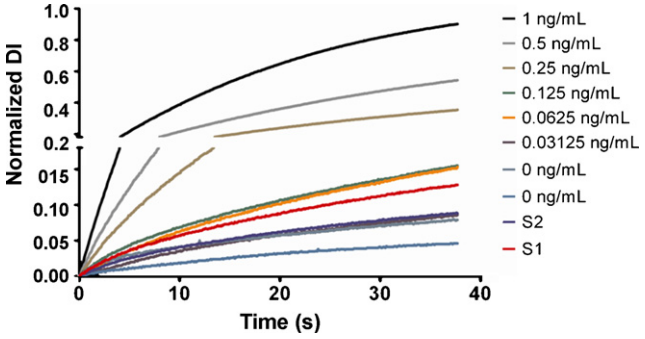
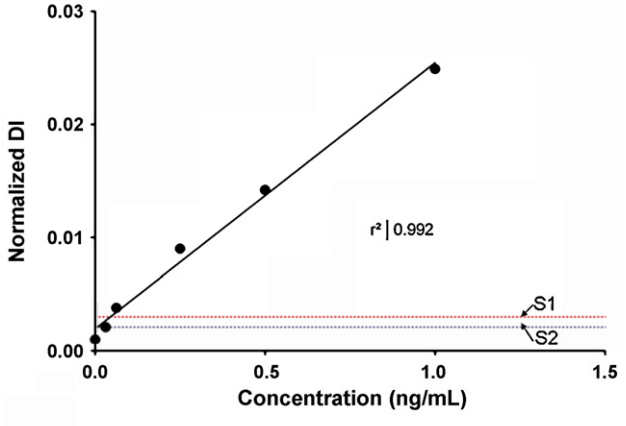


Fig. 10. Overlay of normalized of TMB precipitation zones for the quantitation of residual protein A.
TMB precipitation zones from each calibrator or sample run were normalized to the origin to facilitate comparison (zeroed DI on *y*-axis; zeroed time on the *x*-axis). Precipitation responses are proportional to analyte concentrations. An initial rate determination between time points 5 and 10 s providing reproducible discrimination for each calibrator concentration was selected.



Sample	Derived Value	Dilution Factor	Concentration
S1	0.045 ng/mL	2	0.09 ng/mL
S2	0.007ng/mL*	2	ND

Fig. 11. Calibration curve for residual protein A quantitation.
Initial rates of DI increases between time points 5 and 10 s were plotted against their respective concentrations of calibrator. A simple linear fit was used for the curve. The R^2 value for the fit was 0.9921. The determinations for the 2 unknown samples S1, S2 are indicated on the graph.

in the presence of large concentrations of IgG, the residual assay demonstrated comparable performance to existing techniques for sample 1 (0.094 ng/mL) while for sample 2 the determination was below the detection limit of the current assay (Fig. 11).

4. Conclusions

The dotLab® System's ease of use was demonstrated in the rapid development of several immunoaffinity based assays. As a novel approach to label-free sensing, dot® has some inherent advantages over other label-free optical biosensor technologies. Surface plasmon or waveguide based sensors often require reference channels [5] to eliminate false positive responses. Since the evanescent field in these systems extends several hundreds of nanometers into the dielectric medium, the response is not only dependent on analyte concentration but also on bulk refractive index variations caused by temperature or pressure fluctuations [5]. dot is relatively impervious to these changes as only events that specifically alter the grating will impact the signal. As evidenced by the assays presented in this report, vigorous mixing fluidic routines can be used without any detrimental impact on signal generation. We have also demonstrated the use of this technology in complex media such as serum [4] and whole blood (data not shown) which pose particular issues with technologies that are sensitive to refractive index changes and non-specific surface binding [6]. Other advantages reflect some important design considerations: for instance, the multiplex mode adds an increased measure of reliability providing triplicate determinations for individual assays. The ability to work both in label-free and amplified modes extends the range of detectable analytes on this optical biosensor system and its low cost disposable sensors make it attractive for routine and single use analyses. As for assay development, other immunoassays techniques such as endpoint ELISA's typically comprise a number of steps and utilize a variety of reagents. Since no signal is generated until the last detection step, assay developers must optimize reagent concentrations and steps (time, incubation temperature) through a large number of titration series and experiments, commonly known as "chessboard" or "checkerboard" titrations [7]. Since most of these detection processes are destructive, i.e. the initial capture reagents cannot be reused, assay developers may have to halt development until more reagents are available or until they can justify undertaking this cumbersome process. In short, real-time analysis based on self-referencing dot provides design flexibility by allowing the user to define assay parameters, either in terms of specific time points or rates, and without having to rely on time consuming and labor intensive development of endpoint assays.

Acknowledgments

Sonny Mark, Karen Keith for helpful discussions, Wei Hu and Galina Bernstein for helpful discussions and development of the residual protein A protocol.

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