# Versatile and Customizable Multiplexed Assays Using Optical Diffraction

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## Background

Researchers conducting multivariate diagnostic studies of protein biomarkers benefit from the simultaneous, quantitative measurement of multiple analytes. The ability to detect multiple analytes from high micromolar to low picomolar levels in a single assay with little or no pre-processing of small volume complex biological samples greatly enhances clinical utility. Diffraction-based sensing permits the continuous observation of real time molecular interactions and can significantly accelerate the development and validation of biomarker panels. Diffractive optics technology provides a tool for more informed decisions in assay development and biomarker detection and characterization.

## The dotLab® mX System

The dotLab® mX System utilizes diffraction-based optical sensing for the real time, label-free measurement of molecular interactions. The system uses inexpensive, disposable biosensors with coupling reagents (eg: avidin, amine reactive substrates or unique oligonucleotide-based addressing reagents to allow multiplexing) pre-patterned on the surface of 10 µL flow channels forming a diffraction grating. The dotLab mX instrument illuminates the grating with a laser generating a diffraction image which is monitored by a photodiode detector. Diffractional efficiency increases as molecules bind to the surface resulting in an increase in image intensity. Conversely, molecular dissociation from the surface results in a decrease in image intensity. Therefore, the real time monitoring of molecular interactions through changes in diffractional efficiency provides information on the quantity and rate of binding and dissociation events. The dotLab® mX System simplifies and automates this analysis using a fully integrated, easy to use, bench top instrument.

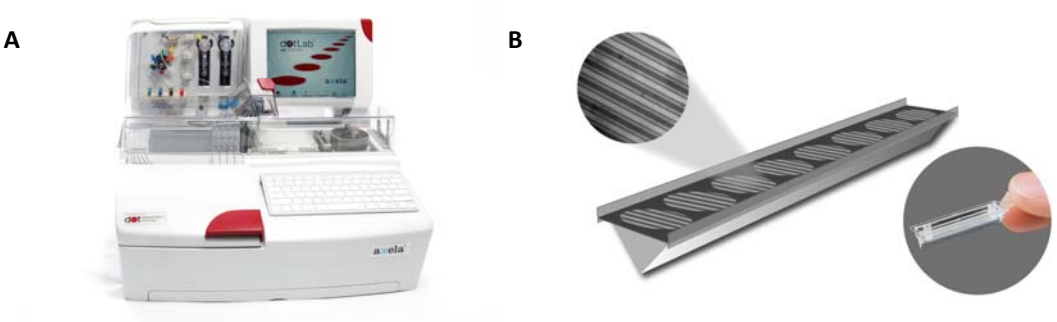



**Figure 1**

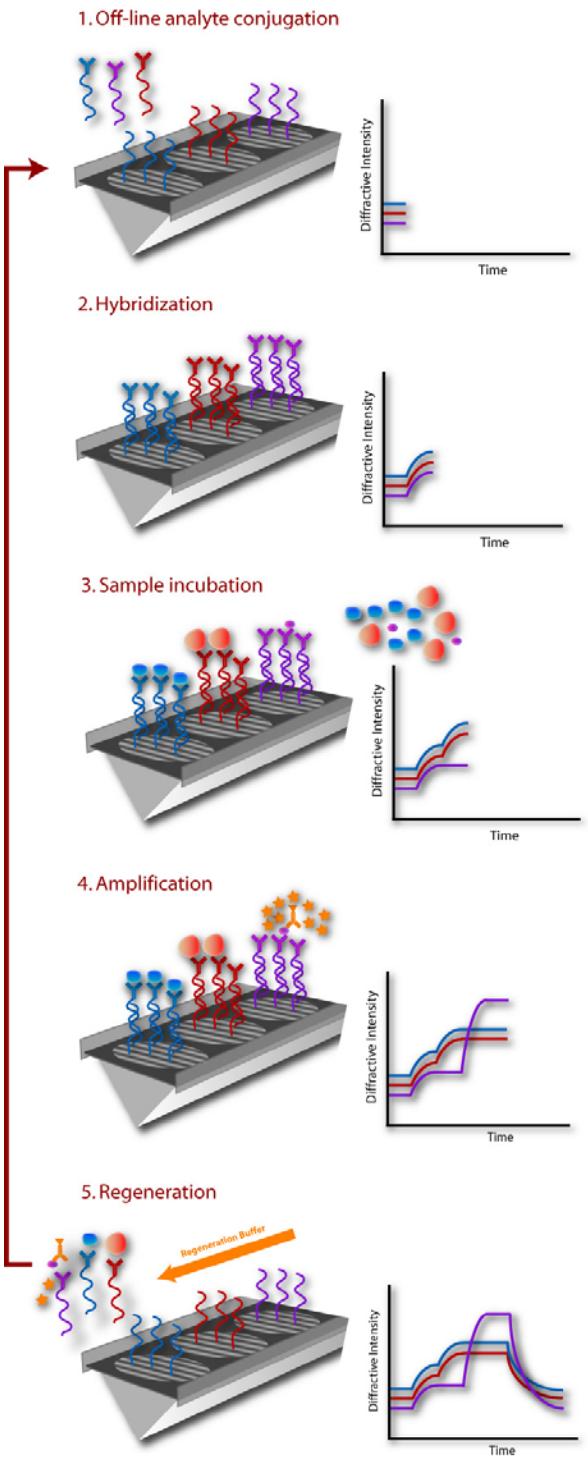
(A) The dotLab® mX Instrument: a fully automated, bench-top instrument for real time molecular interaction analysis. (B) Schematic of a dotLab sensor with a contiguous array of capture surfaces (spots) with coupling reagent pre-patterned on the surface forming diffraction gratings.

In this whitepaper, we demonstrate the use of panelPlus™ Sensors in the development of a multimarker panel and the detection of high and low abundance analytes in a single assay.

## *panelPlus™ Multiplex Technology*

**Figure 2**

Schematic representation of the steps involved in a hypothetical triplexed assay performed on the dotLab® mX System.




Each spot on a panelPlus™ Sensor is coupled to a unique oligonucleotide-addressing reagent. User selected capture reagents can easily be coupled to free oligonucleotides using Axela's Labeling Kit. Alternatively, capture reagents can be selected from an available library of pre-conjugated reagents from Axela.

When the oligonucleotide-conjugated capture reagents are introduced to the sensors, they hybridize to the appropriate spots containing their complementary strand, creating a customized multiplexed panel. The hybridization can be monitored by real time detection on the dotLab® mX System.

Upon sample introduction to the sensor, each capture reagent binds its unique analyte. Using this method, multiple analytes can be captured from a single sample

The ability to sequentially add reagents enable the signal amplification of one or more analytes. This allows a wide detectable dynamic range useful for measuring high and low abundance analytes within a sample.

At the end of an assay, the sensor can be regenerated back to the original surface. This allows cycling of the sensor surface for re-use with the same reagents or the creation of a new panel.

### 1. Development of a Triplex Ovarian Cancer Research Panel

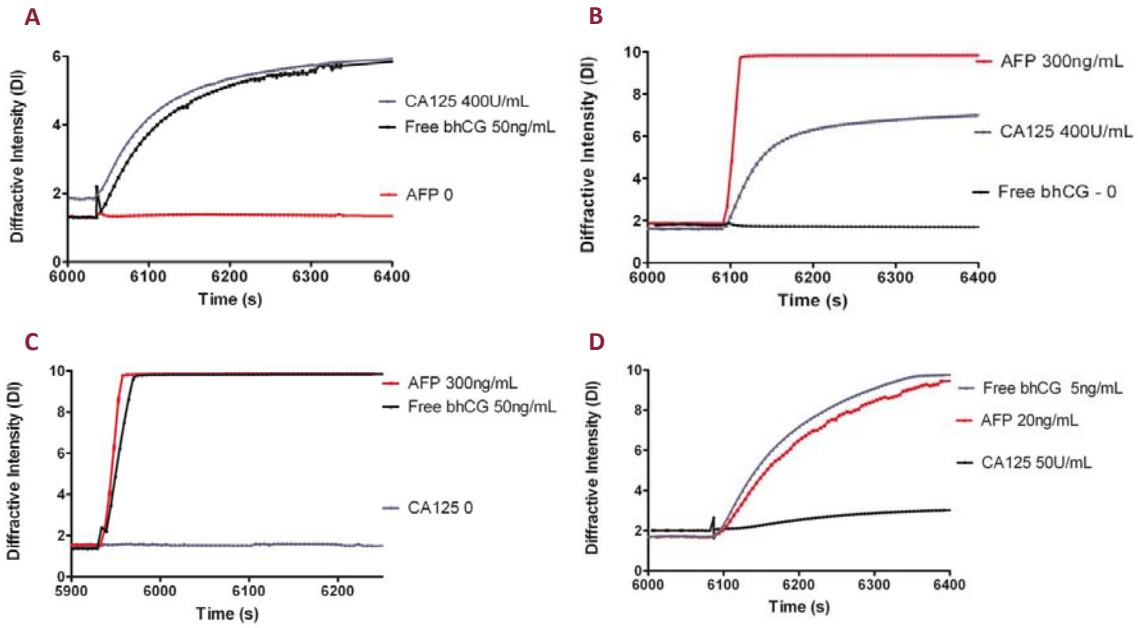
A multiplexed assay was developed to target three biomarkers of ovarian cancer, CA-125, beta human chorionic gonadotropin (bhCG) and alpha-fetoprotein (AFP). Antibodies to each target were conjugated to unique oligonucleotides that are complementary to oligonucleotides addressed at three different spots on a panelPlus™ Sensor. When applied to the sensor, each of the antibody conjugates hybridized to their appropriate spots creating a multiplex capture surface. To test for potential cross talk between

the constituent assays, combinations of test samples containing high concentrations of two of the three biomarkers were applied to one of the ovarian cancer triplex sensors (Figure 3A-C). The absence of signal on the spot of the missing analyte was indicative of no cross talk. In Figure 3A, a sample containing high concentrations of CA125 (400 U/mL) and free bhCG (50 ng/mL) but no AFP did not generate a signal on the spot for AFP suggesting that CA125 and free bhCG do not bind the immobilized anti-AFP antibody. Similar results were observed for anti-free bhCG and anti-CA125 antibodies (Figures 3A and 3B respectively), demonstrating the absence of cross talk between these three assays.

Figure 3D summarizes the analysis of a test serum sample on the ovarian cancer panelPlus™ Sensor. The results show the simultaneous detection of AFP, CA125 and free bhCG from this sample in a single assay.

**Figure 3**

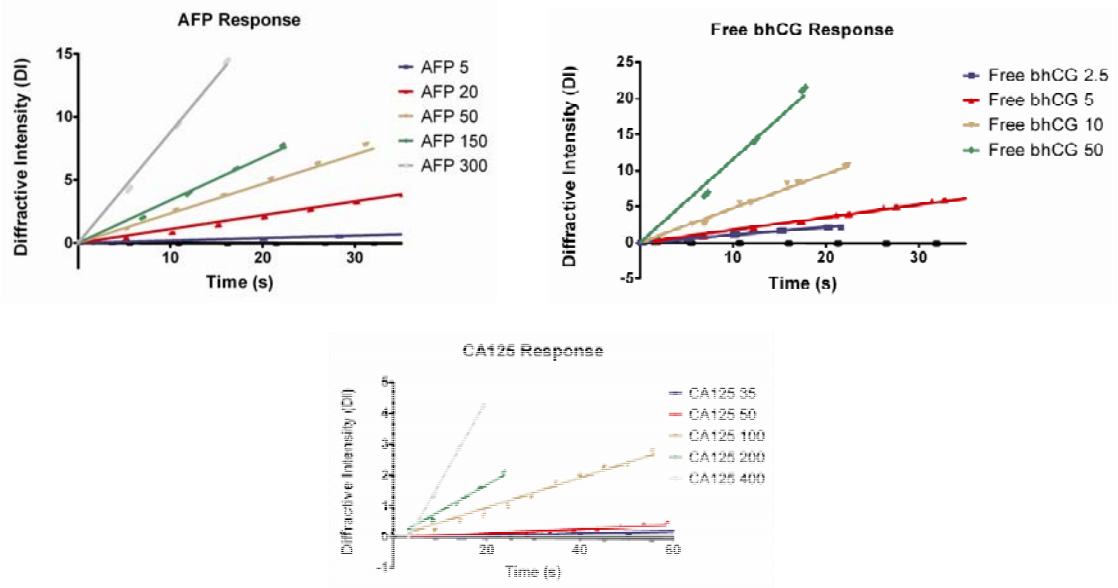
Ovarian cancer triplex assay development and execution. Panels A-C demonstrate the absence of cross talk between the constituent assays. Panel D demonstrates the simultaneous detection of AFP, CA125 and free bhCG from a test serum sample in a single assay.




To generate quantitative results, various concentrations of each of the three analytes, covering their respective clinically relevant range, were spiked into serum and analysed on the triplex sensors. Figure 4 displays the real time response curves for each concentration of analyte. Either the initial slope of the curve or signal intensity after at a defined time point can be used to measure analyte quantity. This data can be used to generate calibration curves for each analyte which in turn can be used to deduce their absolute concentrations in test samples.

**Figure 4**

Quantitative multiplexed assays. Each analyte was analysed at various known concentrations to generate standard curves. Absolute analyte concentrations from unknown samples were then extrapolated from these curves.



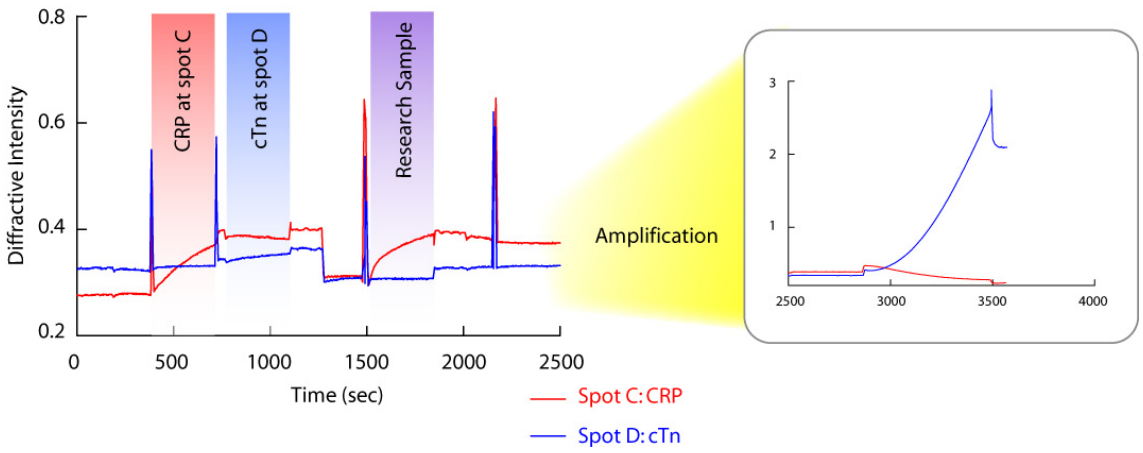
## 2. Extended Dynamic Range: CRP and cTn Duplex Assay

Analytes that are present in high and low abundance in a sample are difficult to measure in multiplexed assays since their signals do not both fall within the linear range of detection at a single dilution. Therefore, multiple sample dilutions need to be analysed to obtain quantitative results. The ability of the dotLab® mX System to sequentially add reagents allows for the addition of analyte-specific amplification reagents. This enables the selective amplification of each analyte until a signal within its linear range of detection is generated in a single assay without the need for multiple analyses at different sample dilutions.

In this example, a duplex assay was performed on C-reactive protein (CRP), a protein that is present in high abundance, and cardiac troponin (cTn), a low abundance analyte (Figure 5). The capture antibodies for CRP and cTn were immobilized on a panelPlus™ Sensor. Loading was performed in separate steps to demonstrate specific binding to their respective spots without cross contamination. Upon introduction of the serum sample, CRP binding was directly detected due to its high abundance while no signal for cTn was observed. cTn binding was then amplified using an alkaline phosphatase (AP)-linked anti-cTn detector antibody and the AP substrate BCIP/NCT. Amplified cTn was generated without spurious signal on the spot for CRP.

**Figure 5**

Detection of high and low abundance analyte in a single assay. CRP (high abundance) and cTn (low abundance) were detected within their respective linear range of detection in a 2-plex assay using panelPlus sensors. This is enabled by the ability to sequentially add analyte-specific amplification reagents to the sensors on the dotLab® mX System.

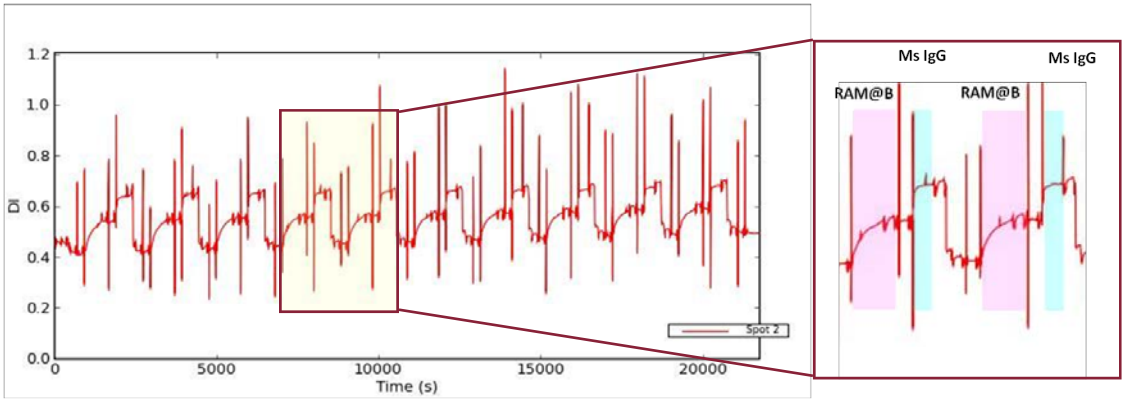



## 3. Sensor regeneration

A significant advantage of the panelPlus™ Sensor is the ability to regenerate the surface following an assay which allows for the continuous re-use of the sensor. Regeneration is performed by using a proprietary buffer which dissociates the oligonucleotide-conjugated capture reagent from its complementary strand on the sensor, creating a fresh surface for subsequent reagent immobilization. The dissociation conditions are identical for all oligonucleotide pairs used in panelPlus-based assays, eliminating the need to optimize regeneration conditions for each target. Figure 6 demonstrates the repeated regeneration of a panelPlus sensor following a simple antibody capture assay where an ol gonucleotide-

**Figure 6**

Regeneration of panelPlus™ Sensors. An oligonucleotide-conjugated rabbit anti-mouse antibody was immobilized on a sensor and used to capture mouse IgG. A regeneration buffer was added to dissociate and remove the complex from the sensor allowing for fresh reagent to be immobilized. The assay was repeated 10 times without significant change in assay performance or baseline signal.



conjugated rabbit anti-mouse antibody is used to capture mouse IgG. The results showed no significant change in assay performance or baseline signal over 10 regeneration cycles. Sensor regeneration allows for the unattended multiplexed analysis of many samples with decreases inter-assay variability, reduces costs and increases overall throughput.

## Summary and Conclusions

- panelPlus™ Sensors allow for the simple development and implementation of user-defined customized multiplexed assays.
- Sequential probing and flexible assay formats provide an extended dynamic range, allowing for high and low abundance analytes to be measured within their respective linear range of detection at a single sample dilution.
- Sensor regeneration enables the unattended multiplexed analysis of a large number of samples and reduces inter-assay variability and cost per assay.

## About Axela, Inc.

Axela's platforms provide powerful new approaches to multiplexed protein and nucleic acid analysis designed to greatly simplify biomarker testing in clinical research and diagnostics. Axela's commercial research products significantly improve the amount and quality of information derived from traditional assays. This approach shortens time to result and provides access to unique categories of markers that form a pipeline of future diagnostic offerings.

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