Application Note

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Detection of Human IgG Using a Luminescence-Based Proximity Assay

Using spatial proximity analyte reagent capture luminescence (SPARCL) assays

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Abstract

Homogeneous proximity assays allow the quantitation of specific analytes from a complex matrix containing numerous contaminants without the need to use wash steps. Because these assays are performed in solution without binding to a solid surface, reaction kinetics are more rapid than conventional ELISA-based assays. This application note describes the use of the Agilent BioTek Synergy Neo multimode reader in conjunction with spatial proximity analyte reagent capture luminescence (SPARCL) assay technology to quantitate human IgG.

Introduction

Biomedical research requires that numerous analytes be quantitated, often in large numbers of experimental samples. Towards that end, a number of different homogeneous proximity assays have been developed to quantitate specific analytes from a complex sample matrix without the need for wash steps to remove unwanted contaminates. Eliminating the necessity to remove unbound contaminates not only saves considerable time, but also reduces the amount of instrumentation needed to automate the assays. These assays share a common feature in that signal is only generated in the presence of analyte, resulting in low background signal and excellent S/B ratios. As the name implies, this is accomplished by bringing two molecules together in close enough proximity such that an energy transfer can take place, with the analyte serving as the bridge. Typically, antibodies serve as the means for analyte specificity. These features make them the preferred assay type for many screening type experiments.

A number of different homogeneous proximity assays have been developed. For example, the scintillation proximity assay (SPA) depends on the close proximity of a radio-labeled molecule to a bead containing a scintillant for signal.[1] Bioluminescence resonance energy transfer (BRET) requires that a bioluminescent moiety be close enough to a green fluorescent protein molecule to allow the transfer of energy.[2] Time-resolved fluorescence resonance energy transfer (TR-FRET) and HTRF require that two fluorescent molecules be in close proximity, with the donor fluor having an exceptionally long decay half-life.[3,4] Amplified luminescent proximity homogeneous assay (ALPHA) screen assays use donor beads that generate singlet oxygen that will interact with acceptor beads located within 200 nm to generate a chemiluminescent signal.[5]

There are also several nucleic acid-based proximity assays. PINCER technology uses fluorescently labeled complimentary single stand nucleic acids attached to antibodies.[6] Proximity ligation assays (PLAs) are based on the amplification of a DNA sequence, which is dependent on the proximity of two antibodies labeled with different oligonucleotides. They form a circular DNA molecule with the help of a connector oligonucleotide, which can be detected by rolling circle amplification.[7] Proximity extension assays use target-specific antibody pairs linked to DNA strands that, upon simultaneous binding to the target analyte, create a real-time PCR amplicon in a proximity-dependent manner enabled by the action of a DNA polymerase.[8]

Spatial proximity analyte reagent capture luminescence (SPARCL) technology is a proximity-dependent, chemiluminescent detection technique. In the assay, a chemiluminescent substrate (acridan) is brought into the proximity of an oxidative enzyme (horseradish peroxidase, HRP) through the specific antigen/antibody interaction (Figure 1). A flash of light proportional to the quantity of analyte present in the sample is generated upon addition of a trigger solution containing hydrogen peroxide (H_2O_2) and *para*-hydroxycinnamic acid (pHCA) enhancer. Heterocyclic compounds such as pHCA have been shown to increase light emission from HRP-based luminescent reactions greater than 1,000 fold.[9,10] Data from similar reactions with luminol have resulted in the proposal that enhancers serve as a conduit for the transfer of free radicals from HRP to the luminescent agent (i.e. acridan).[11] This application note presents data demonstrating SPARCL technology, a homogeneous assay based on a bridging assay format where all reagents and sample are in solution.

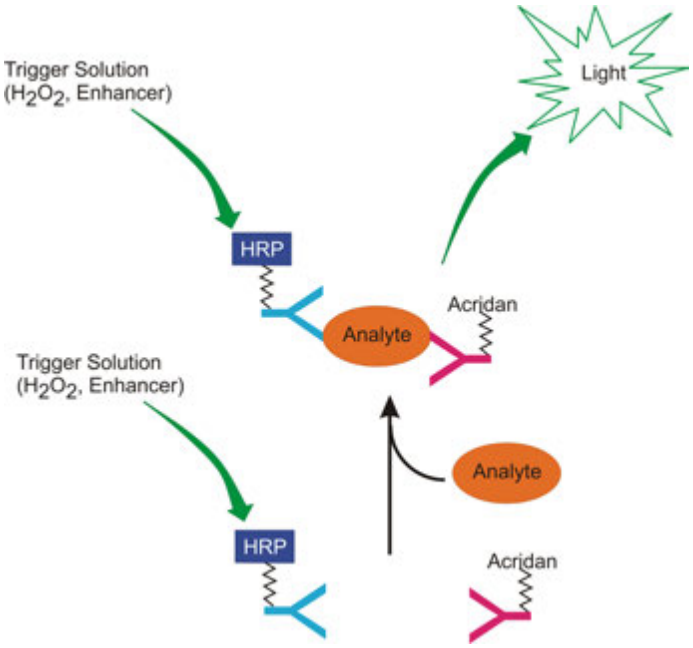

Figure 1. SPARCL reaction. The oxidative enzyme HRP is in close proximity with the chemiluminescent substrate acridan only when both antibodies are bound to the analyte.

Horseradish peroxidase (HRP) catalyzes the hydrolysis of H_2O_2 and in the process generates free radicals, which can oxidize substrates that are nearby. However, these reactive molecules have a limited lifespan and can only diffuse short distances before they are destroyed by interacting nonspecifically with proteins or lipids. Acridan molecules in

close proximity can serve as such a target for attack by the newly generated free radicals. In doing so, acridans will form a dioxetone intermediate that decays to an excited acridone molecule that will emit light photons as it returns to its stable state (Figure 2). Because free radicals are highly reactive, only acridan molecules in close proximity to the source of free radicals will result in the emission of specific light photons. Only when an analyte has both a HRP conjugate and an acridan conjugate bound will the two moieties be in close enough proximity for the reaction to take place. Unbound antibodies in solution will not result in the necessary reaction proximity for a luminescent reaction to occur.

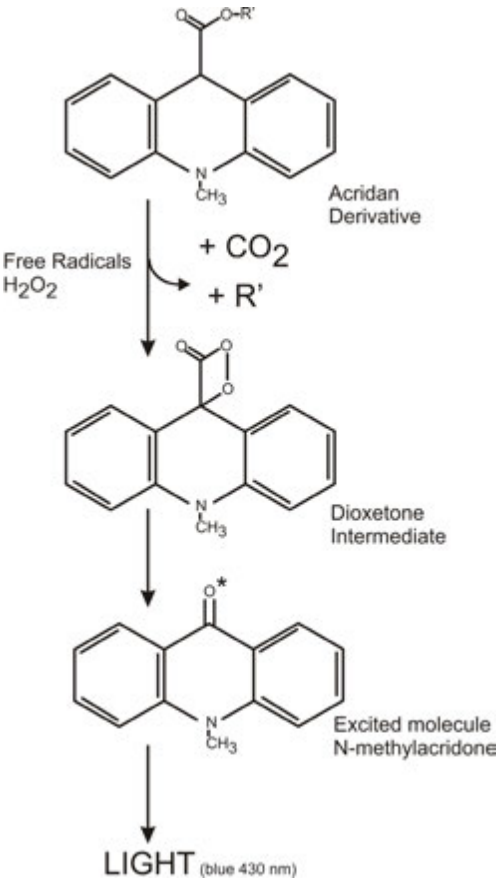

Figure 2. Acridan chemistry. An example of acridan chemistry where reaction with free radicals results in the emission of light photons.

Materials and methods

SPARCL assay reagents including acridan-labeled JDC-10 antibody, HRP-conjugated JDC-10 antibody, human IgG, Trigger solution, and background reducing agent were provided by Lumigen (Southfield, MI). Normal Rat serum (part number 10710C) was obtained from Life Technologies (Carlsberg, CA) and solid white 96-well microplates (part number 3912) and plates seals (part number 6569) were from Corning Life Sciences (Tewksbury, MA).

SPARCL proximity assays were set up as follows. Working solutions of HRP and acridan labeled antibodies were made the day of the assay. HRP-conjugated JDC-10 antibody stock (50 µg/mL) was diluted 1:800 and acridan-labeled JDC-10 antibody stock (500 µg/mL) was diluted 1:78 with assay buffer (PBS with 0.1% BSA). Human IgG stock (10 mg/mL) was diluted to make a series of standards (0 to 4,000 ng/mL) using normal rat serum as the diluent.

Equal amounts of HRP and acridan-labeled antibody working solutions were combined and 50 µL of the mixture was aliquoted into the wells of the microplate. Samples and standards (25 µL) were then pipetted into wells of the microplate and the plate was sealed with an aluminum adhesive sealer, and incubated on a plate shaker for 30 minutes at room temperature. After incubation, 4 µL of background reducing agent was added to each well and the plate was positioned on the plate reader. Luminescence was determined using an Agilent BioTek Synergy Neo multimode reader. Reactions were initiated by the injection of 75 µL of trigger reagent using the reader's reagent injectors. The reactions were measured kinetically in well mode every 0.02 seconds for a total of 2 seconds per well. Sensitivity was set to 175 and the data integral recorded (Figure 3).

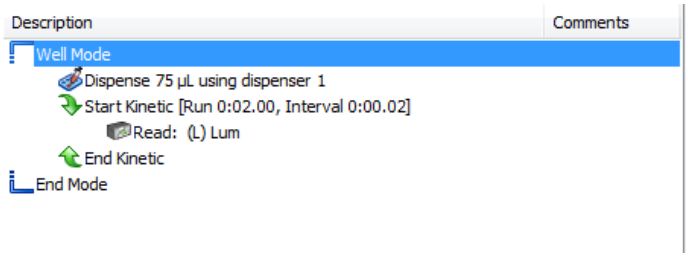

Figure 3. Dispense and read process. The luminescent signal is captured over 2 seconds after dispensing of trigger/enhancer reagent.

Results and discussion

Kinetic luminescence data were captured using Agilent BioTek Gen5 data analysis software. When the mean signal at each time point is plotted for three different human IgG concentrations (2,000, 300, and 0 ng/mL), marked differences in the plots are observed. Samples containing IgG have substantial signal that diminishes over the kinetic interval, while the 0 ng/mL sample has virtually no signal (Figure 4). These data also show how rapid the luminescent reaction is.

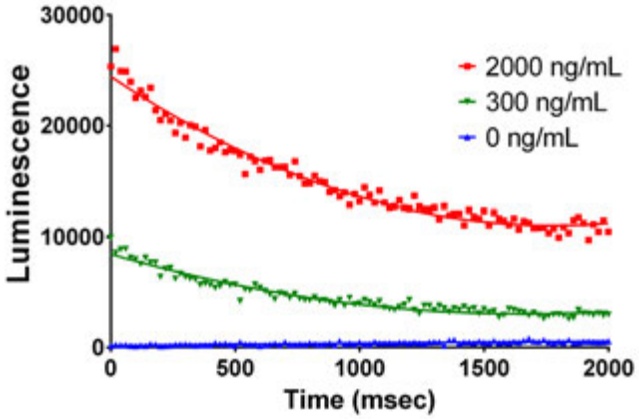

Figure 4. Kinetic luminescence of SPARCL assay for IgG at different concentrations. Kinetic data was captured every 20 msec for a period of 2 seconds. Each data point represents the mean of 10 determinations with a second polynomial regression analysis.

When the integral or area under the curve is calculated and plotted, a linear relationship between analyte concentrations is observed (Figure 5). This relationship can be described using a 4-parameter logistics fit of the data, with a Z'-factor value in excess of 0.9 between the 0 and 1,500 ng/mL standards.

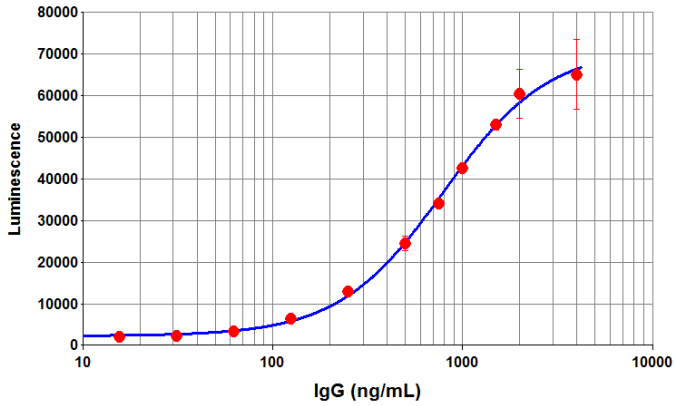

Figure 5. SPARCL IgG titration curve. Various concentrations of human IgG were assayed using SPARCL reagents specific to IgG. Data represents the mean and standard deviation of the integral of kinetic luminescent measurements for 4 replicates.

Conclusion

This application note shows that the Agilent BioTek Synergy Neo multimode reader is capable of making the luminescent determinations necessary for SPARCL assays. The Synergy Neo, as well as other Agilent BioTek multimode readers, provide a number of features that make them amenable to rapid kinetic flash-type luminescent assays. Foremost is the optional reagent dispenser module that allows automated dispensing of two different reagents into the wells of the microplate while the plate is located in the read chamber. This feature allows the addition of trigger reagent and enhancer and almost immediate detection of the luminescence. Agilent BioTek Gen5 data analysis software controls reader functions, collects and stores generated data, and performs data reduction. A number of different curve fits are available to describe data or to be used as standards curve for interpolation to calculate unknown sample concentrations.

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