

## Determination of IL-6 Cytokine Levels by ELISA with Resonance Raman Signal-based Peroxidase Detection

Use of Sword Diagnostic's Peroxidase Reagents with the Synergy™ H4 Microplate Reader



Paul Held Ph. D., Applications Department, BioTek Instruments, Inc., Winooski, VT  
 Charles Ginsburgh Ph. D., Sword Diagnostics, Chicago, IL



Enzyme Linked Immunosorbent Assay (ELISA) technology has been one of the mainstays of biomedical research for the last 40 years. The assay technology employs antibodies for specificity in conjunction with a chromogenic substrate for the detection and quantification of analytes. Reduction in analyte detection limits has been driven through the use of better antibodies, improvement in the reporter enzyme substrates employed in the assay, and the use of more sensitive instrumentation. Here we describe the use of the Synergy H4 Hybrid microplate reader to detect and quantify analytes using resonance Raman signal generated with Sword Reagent peroxidase substrate.

### Principles of the ELISA Assay

This assay employs the quantitative sandwich enzyme immunoassay technique. A microplate is pre-coated with a purified antibody specific for murine IL-6. Standards and samples are pipetted into the wells and any IL-6 present is bound by the immobilized antibody. After washing away any unbound substances, a mixture of biotinylated anti-mouse IL-6 detection antibody and streptavidin-horseradish peroxidase conjugate solution is added to the wells. After performing a second wash cycle, a substrate solution is added, which reacts with the bound peroxidase enzyme to produce a signal. The magnitude of this signal is directly proportional to the concentration of murine IL-6 present in the original specimens added.

### Sword Diagnostics Reagents

The detection system uses a peroxidase substrate that produces a product capable of Raman excitation. Stabilized hydrogen peroxide and 5-aminosalicylic acid at pH 6.5 is converted to an iminoquinone by the enzymatic action of horseradish peroxidase. The subsequent addition of sodium hydroxide reagent enables the production of a resonance Raman signal [1].

### Materials and Methods

Medium binding (#2693) and high binding (#2692) stripwell microplates were obtained from Corning (Corning, NY). Mouse IL-6 Assay reagents, obtained as an OptEIA reagent set (cat # 555240, lot 78698), was obtained from BD Biosciences (Franklin Lakes, NJ). The reagent set contains recombinant Mouse IL-6 Lyophilized Standard (#51-26536E), capture Antibody Purified Anti-Mouse IL-6 (# 51-26531E), detection Antibody Biotin Anti-Mouse IL-6 (# 51-26532E), and enzyme Reagent Streptavidin-horseradish peroxidase conjugate (# 51-9002813). Additional assay reagents obtained from BD Bioscience (as OptEIA™ Reagent Set B - Cat. No 550534), containing coating buffer (0.1 M Sodium Carbonate, pH 9.5); assay diluent (PBS with 10% FBS, pH 7.0); 20x wash buffer (#51-9003739); substrate solution (Tetramethylbenzidine TMB and Hydrogen Peroxide); and stop solution (2N H<sub>2</sub>SO<sub>4</sub>) was also used. The Sword Diagnostics Peroxidase Reagents (catalog # N818) was obtained from Sword Diagnostics (Chicago, IL). An ELISA for the mouse IL-6 cytokine was then developed as described below (Figure 1).

### Key Words:

ELISA  
 Resonance Raman Emission  
 Fluorescence  
 Quantitation

### Microplate Coating

Capture antibody was first diluted 1:250 using coating buffer and 100  $\mu\text{L}$  was added to each well of 96-well stripwell microplate. Plates were either incubated overnight at 2-8  $^{\circ}\text{C}$  or at room temperature for 120 minutes with mixing using an orbital shaker. Coated plates were then washed four times (350  $\mu\text{L}$ /well) with 1x wash buffer previously prepared by dilution of a 20x concentrate with deionized water. After washing, plates were inverted and blotted on absorbent paper to remove any residual buffer.

### Microplate Blocking

Coated plates were blocked using assay diluent (250  $\mu\text{L}$ /well) for 60 minutes at room temperature. After blocking plates were washed four times (350  $\mu\text{L}$ /well) with 1x wash buffer previously prepared by dilution of a 20x concentrate with deionized water. After washing, plates were inverted and blotted on absorbent paper to remove any residual buffer.

### Assay Procedure

A series of standards were made ranging from 0 to 200  $\mu\text{g}/\text{mL}$  concentration. Aliquots (100  $\mu\text{L}$ ) of each dilution were pipetted into the previously prepared assay plate. The reactions were allowed to incubate at room temperature for 120 minutes, after which they were washed using BioTek's ELx50™ Strip Washer eight times with 350  $\mu\text{L}$ /well of wash buffer. After the last wash the plates were inverted and blotted on absorbent paper to remove residual fluids. Working detector reagent (100  $\mu\text{L}$ ), consisting of equal portions Detection antibody solution and HRP conjugate solution, was then added and allowed to incubate for 60 minutes at room temperature. After completion of the incubation, plates were then washed again 8 times as described previously. Development steps varied, depending on whether Sword substrate/peroxidase mixture or TMB substrate was used. With the Sword substrate, 150  $\mu\text{L}$  of Sword substrate/peroxidase mixture was added to all the wells and allowed to incubate 15 minutes in the dark at room temperature, followed by the addition of 150  $\mu\text{L}$  of development solution. The plate was then incubated for a further 30 minutes in the dark at room temperature prior reading (Figure 1). Wells receiving TMB substrate received 100  $\mu\text{L}$  of TMB substrate and were incubated for 30 minutes, followed by the addition of 50  $\mu\text{L}$  of stop solution.

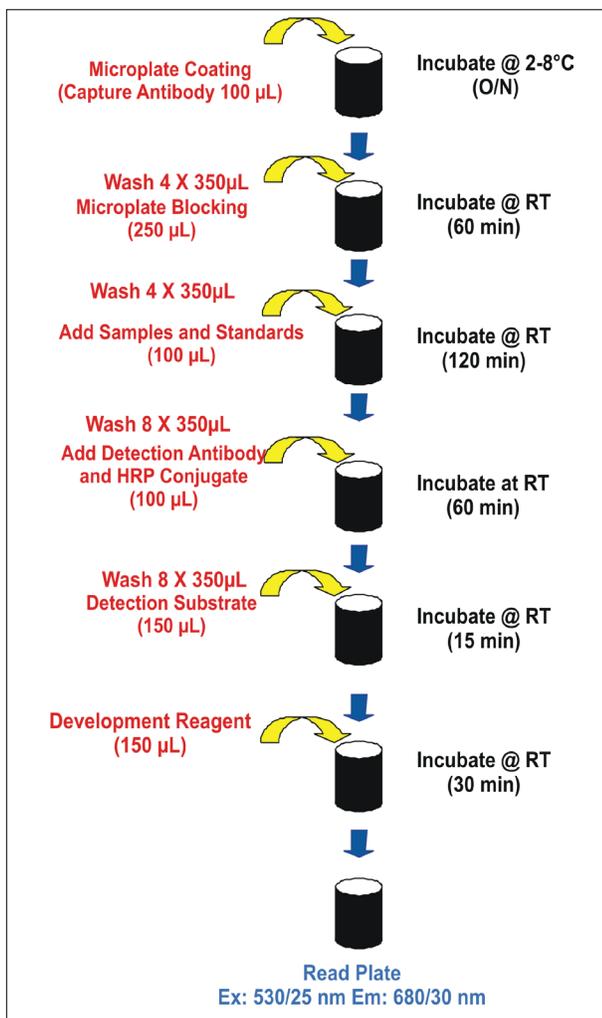


Figure 1. IL-6 ELISA Process.

### Detection

The Synergy H4 Multi-Mode Microplate Reader was used for detection of both TMB and Sword reagent based samples. The absorbance of each well for TMB reactions was measured at 450 nm and 570 nm. The 570 nm result (reference) was subtracted from the 450 nm result (experimental) to correct for background absorbance from the microplate. Resonance Raman signals generated using Sword reagents was measured using fluorescence intensity detection mode from the top of the plate. Wavelength selection was accomplished using either monochromator- or filter-based optics. An extended red PMT was used for detection of the signal.

### Data Analysis

The reader was controlled and data captured using Gen5™ Data Analysis Software (BioTek Instruments). Dose response data was fitted into to a five parameter logistic fit curve (5-P) using the following equation:

$$\text{Eq 1. } y = D + \frac{(A-D)}{\left[1 + \left(\frac{x}{C}\right)^B\right]^P}$$

Where variable y corresponds to the assay response value of a given analyte at a concentration x and A is the theoretical response at the lowest concentration, B is the relative slope of the curve at its inflection point, C is the concentration value at the inflection point, D is the response at the highest concentration and P is the asymmetry of the curve [3].

The analytical limit of detection (LOD) is defined as the concentration determined from the interpolation of the 5-P fit curve at the response level equal to the mean of the negative control plus two times the standard deviation estimated from the negative control data population.

Comparisons of curve shift were made from the half maximal response. The C-parameter of the 5-parameter logistic fit is the concentration at the inflection point or half maximal response. Comparison of the C parameters from different experiments indicates the degree of shift using the Sword reagents.

### Results

Initial spectral analysis experiments were performed to confirm excitation and “emission” peaks. Note that the resonance Raman signal measured as an “emission” is actually not an emission event, but a scattering of a percentage of the incidence light. However this distinction is not germane to the consideration or application of the appropriate emission settings applied here. Using an emission wavelength of 700 nm, a spectral excitation scan of reacted Sword peroxidase reagent was performed using the Synergy™ H4. As demonstrated in Figure 2, the solution has a peak excitation wavelength of 530 nm. This wavelength was then used as the excitation wavelength for an emission wavelength scan. The peak emission wavelength was found to be 670 nm.

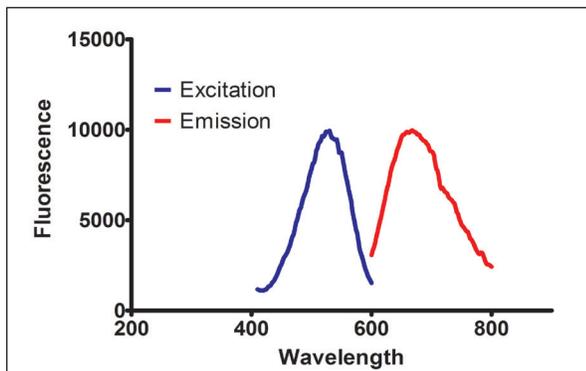


Figure 2. Spectra of Resonance Sword Reagent Raman Emission.

Using this spectral information, the PMT gain and z-height adjustment were optimized for both the filter- and monochromator-based optical paths. Using a series of HRP titrations the effect of optical probe Z-height was determined as measured by Raman emission, Signal to Background ratio (S/B), and %CV. As shown in Figure 3, the Z-height setting was varied from 4 to 12 mm for the filter based optics and 4-11 mm for the monochromator based optics. While the Raman emission signal was maximal at the default setting of 7 for the filter based optics respectively, slightly better signal to background ratios were observed with a z-height setting of 10 mm. The monochromator based optics seems to have improved performance with increasing Z-height. The precision of measurements from replicate samples, as indicated by %CVs was around 1% for the filter based measurements and averaged 5% for monochromator based determinations.

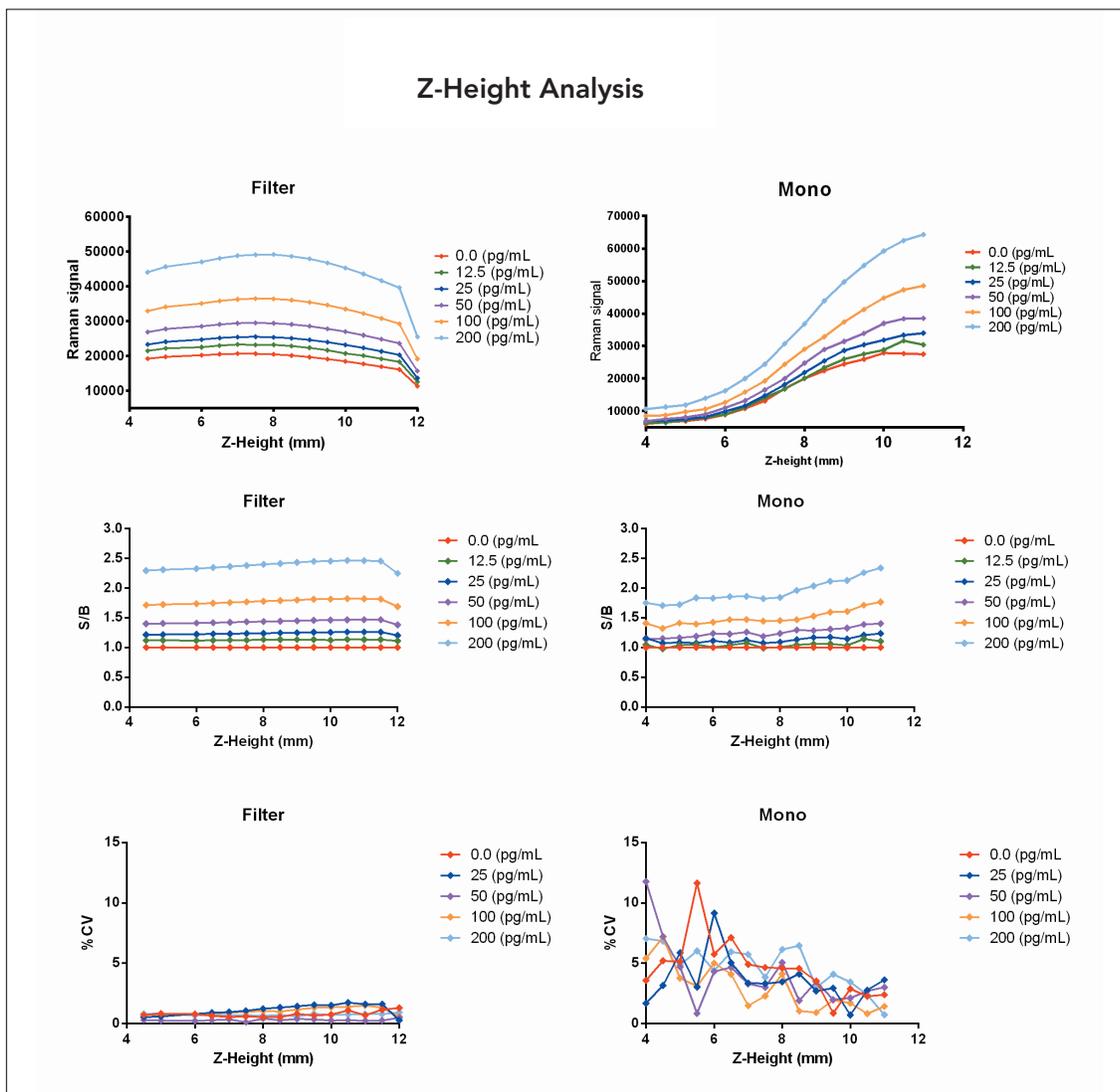


Figure 3. Optimization of top probe Z-height with Mono and Filter Optical Paths.

PMT gain can be used to increase the measured Raman scatter signal of the samples. As with any instrument measurement it is important that the signal be within linear range of the detector. Using a fixed Z-height setting the PMT gain was varied for both the filter- and monochromator-based optical systems. Not surprisingly, increasing the PMT gain setting increased the Raman signal output (Figure 4). However the S/B ratio did not change indicating that the instrument signal was linear. Regardless of the gain setting utilized, the filter based optics returned a %CV of 2% or less, with little change regardless of the gain setting. The monochromator optics was more variable, on average the %CV for the monochromator optics ranged from 2-6%.

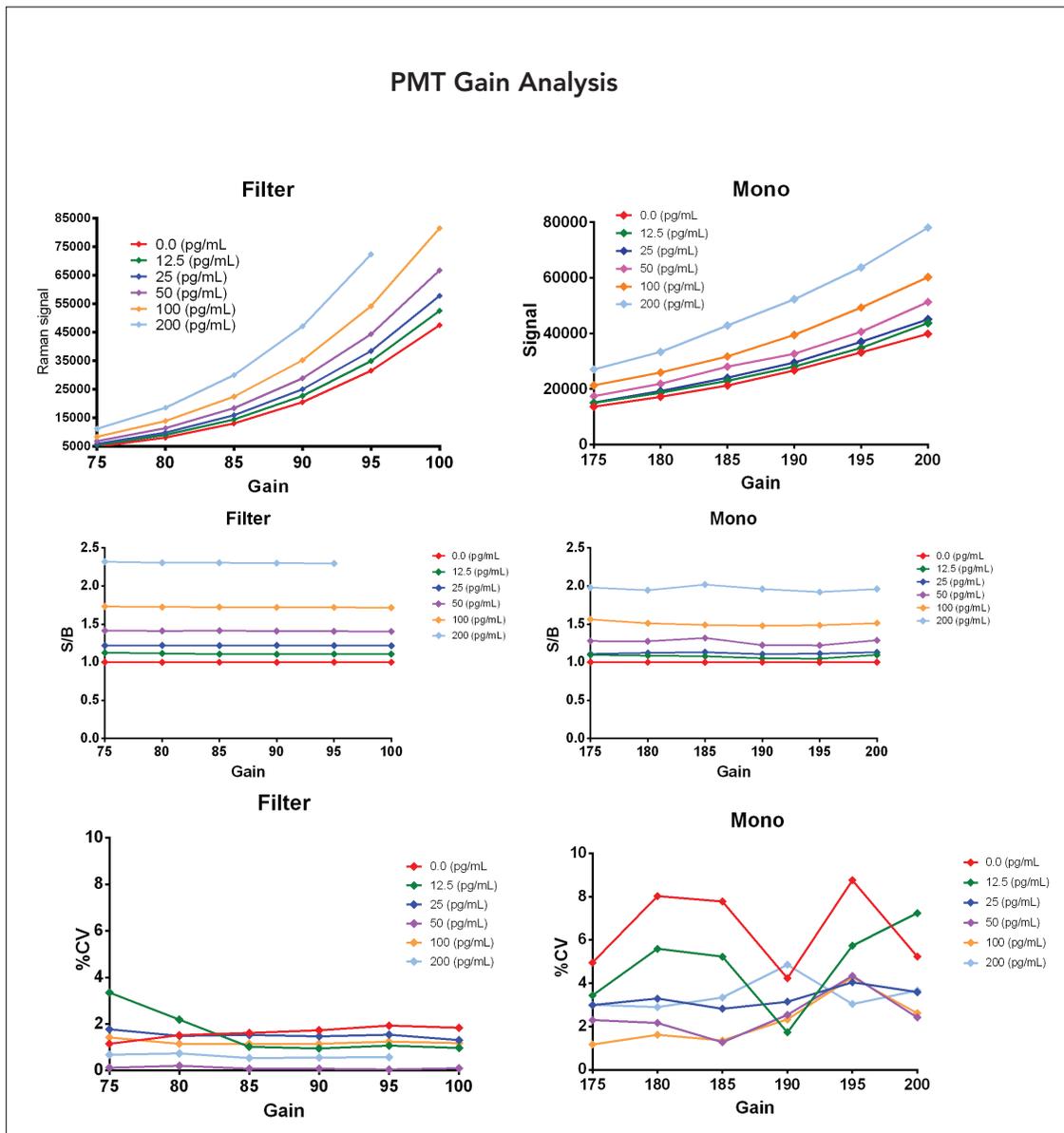


Figure 4. Optimization of PMT Gain for Mono and Filter Optical Paths.

The Synergy H4 is a modular Hybrid Multi-Mode Microplate Reader that can be configured with either filter- and/or monochromator-based optics in the system. Both optical systems provide good performance with ELISA assays (Figure 5). When one compares the % maximal performance of Raman emission plots of IL-6 ELISA reactions, very similar plots are observed with either filter- or monochromator-based optics. Both the Sword diagnostic reagent and TMB are converted using the conjugated peroxidase enzyme HRP, making side by side comparisons possible by replacing one substrate for another. Absorbance-based detection of the same ELISA assay produces a curve that is shifted rightward as compared to Raman emission substrates. The result is that the Raman emission based substrate provided by Sword Diagnostics has a lower limit of detection (LOD), along with substantially lower EC<sub>50</sub> values.

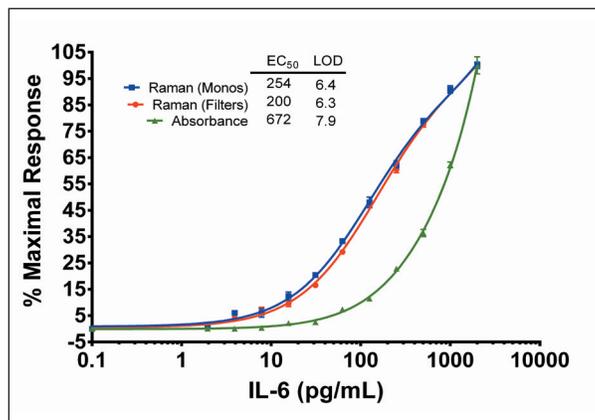


Figure 5. Comparison of Sword Peroxidase reagents and TMB IL-6 Dose response curves.

In addition there is a greater signal response at the lower concentrations with Sword peroxidase substrate than TMB. The net result is a significant increase in sensitivity with the assay through a substitution of substrates.

## Discussion

These data demonstrate that the Synergy H4 Multi-Mode Microplate Reader can be used in conjunction with the Sword peroxidase reagents to detect 3-4-fold lower IL-6 sample concentrations than conventional absorbance at the half-maximal response. This is observed as a significant shift toward lower analyte concentrations. While the limit of detection (LOD) between the two technologies is only moderately better with the Sword Reagents, the reliability of measurement is substantially increased at low concentrations. Significant signal change is occurring across the entire range of concentrations with the Sword peroxidase reagents, while a substantial portion of curve is relatively unchanging with the absorbance based reagents.

The Synergy H4 is a modular instrument and can be configured in a number of different ways. Most importantly for the detection of the Resonance Raman emission produced by the converted product of the Sword Reagent is the use of an extended red PMT (P/N 49721) in lieu of the standard low-noise PMT. The extended red PMT, as its name implies, has an extended wavelength response in the near IR range of the light spectrum, which results in a better signal response. The reader can also be configured with either filter and/or monochromators for fluorescence wavelength selection. While the data presented indicate that either optical system are capable of obtaining reliable and accurate resonance Raman signal measurements, the filter based optics do so by requiring less PMT amplification. This results in less instrument induced variability leading to greater well to well precision.

The Synergy H4 has two different light sources available for excitation, continuous tungsten halogen lamp and a xenon flash lamp. In our tests, only the high energy from the xenon flash bulb was able to adequately produce resonance Raman emission.

This technical note describes the improved performance obtained by substituting the Sword Diagnostics Peroxidase reagents for a conventional TMB substrate. One additional benefit to the leftward shifted Sword dose response curve is that more analyte dependent signal is now present at the lower analyte concentrations. This allows more flexibility to an assay developer in achieving the most advantageous mix of assay performance attributes (such as sensitivity, specificity, precision, kinetics, etc.).

Further improvement would be expected from re-optimization of the ELISA with the new detection system. Improved S/B ratios could be expected by altering the capture antibody or conjugate antibody concentrations of the assay to accommodate the new substrate.

## References

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