APPLICATION NOTE

Detect inflammatory cytokines at picogram levels with Sword ELISA Boosters

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Benefits

- **Highly sensitive measurement of low-abundance analytes**
- **Enhancement of traditional ELISA chemistries with patented Sword molecules**
- **Low background noise and CVs**

Introduction

Traditional ELISA (enzyme-linked immunosorbent assay) methods using the horseradish peroxidase (HRP) substrate 3,3',5,5'- tetramethylbenzidine (TMB) often fail to detect low abundance analytes, such as inflammatory cytokines. Sword Diagnostics has developed the Sword ELISA Booster, a next-generation ELISA detection technology that directly replaces the detection reagents in a traditional, gold-standard ELISA, to increase sensitivity up to 30 fold.

The Sword ELISA Booster uses immunoassay chemistry with the addition of patented detection reagents. The Sword molecule is converted to a resonance Raman-active substrate in the presence of horseradish peroxidase (HRP), and resonance-Raman signal is detected on a fluorescence microplate reader. This enhances performance of the assay, as superior enzyme/substrate interaction at low concentrations produces more resonance-Raman active molecules. The assay enables higher sensitivity, consistently lower CVs, and less noise compared to traditional ELISA detection chemistries.

Here, ELISAs for the inflammatory cytokines TNF-α and IL-1ß were performed using Sword ELISA Boosters and detected using the fluorescence read mode of Molecular Devices SpectraMax® iD3, i3x, and M5 Multi-Mode Microplate Readers. Both analytes were measured at low pg/mL levels on all three readers.

Materials

- Sword ELISA Booster for Human IL-1ß (Sword Diagnostics cat. #SB-HIL1B02-05)
- Human IL-1ß/IL-1F2 DuoSet ELISA (R&D Systems cat. #DY201)
- Sword ELISA Booster for Human TNF-α (Sword Diagnostics cat. #SB-HTNFA02-05)
- Human TNF-α DuoSet ELISA (R&D Systems cat. #DY210)
- Immuno Clear Standard Modules (strip well plates) with Nunc MaxiSorp coating (Thermo Scientific cat. #445101)
- SpectraMax iD3 Multi-Mode Microplate Reader
- SpectraMax i3x Multi-Mode Microplate Reader
- SpectraMax M5 Multi-Mode Microplate Reader
- MultiWash+ Microplate Washer

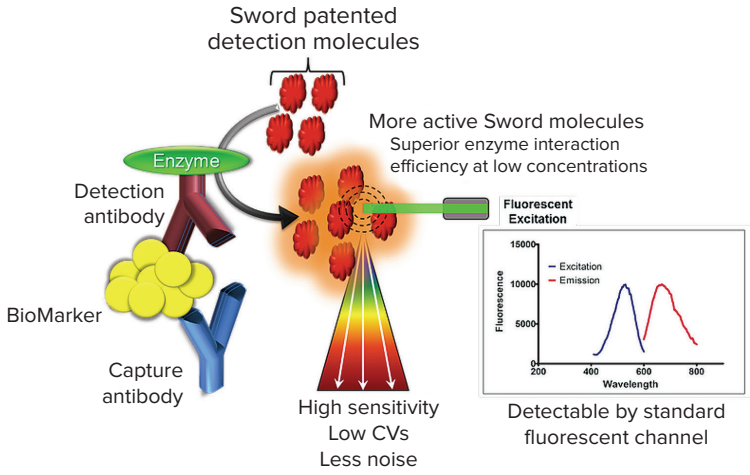

Figure 1. Sword ELISA Booster technology. Patented Sword molecules are detected using a fluorescence reader.

Methods

Preparation of working solutions, plate preparation, and assay procedure for both kits are described below. For additional details on reagent handling and methods, please refer to the product inserts.

Sword ELISA Booster substrate solution

To prepare 16 mL Sword ELISA Booster substrate solution, enough for one 96-well plate, the following were added to 11.2 mL deionized water:

- 1.6 mL Sword Booster Component A (10X)
- 1.6 mL Sword Booster Component B (10X)
- 1.6 mL Sword Booster Component C (10X)

1X Sword Development solution

To prepare 16 mL Sword Development solution, enough for one 96-well plate, 3.2 mL 5X Sword Development solution (Component D) was added to 12.8 mL deionized water.

Plate preparation

IL-1ß and TNF-α capture antibodies were each reconstituted with 0.5 mL of PBS to prepare antibody concentrates. Concentrated Human TNF-α Capture Antibody was diluted to 12 µg/mL in PBS, and Human IL-1ß Capture Antibody was diluted to 4 µg/mL in PBS, to make working concentrations of antibody. Wells of each assay plate were coated with 100 µL/well of their respective diluted antibody. The plates were then sealed and incubated overnight at 4°C.

Using the MultiWash+ Microplate Washer, wells were aspirated and washed three times with 400 µL/well wash buffer (PBS + 0.5% Tween 20), allowing wash buffer to sit in the plate for 15-30 seconds prior to each aspiration. Plates were then blocked with 200 µL/well Sword ELISA Blocker for IL-1ß or TNF-α, sealed, and incubated at room temperature for at least one hour. Aspiration and washes were repeated as above.

Parameter	SpectraMax iD3	SpectraMax i3x	SpectraMax M5
Optical configuration	N/A	Monochromator	N/A
Read mode	FL (fluorescence)		
Read type	Endpoint		
Wavelengths	530 nm Ex 710 nm Em	530 nm Ex 710 nm Em	530 nm Ex 710 nm Em (630 nm Cutoff)
Plate type	96-well standard (optimized)		
PMT and optics	PMT gain: Automatic Integration time: 500 ms Read from top Read height: 1 mm	PMT gain: High Flashes per read: 6 or 100 Read from top Read height: 5.34 mm	PMT gain: Automatic Flashes per read: 100 Read from top

Table 1. SpectraMax reader settings for Sword assay detection. Both SpectraMax iD3 and SpectraMax M5 readers use monochromator-based optics, so there is no need to select an optical configuration in the settings.

Plate reader	IL-1ß LLOQ (pg/mL)	TNF-α LLOQ (pg/mL)	IL-1ß EC_{50}	TNF-α EC_{50}
SpectraMax iD3	0.41	0.98	54.6	78.9
SpectraMax i3x	0.41	0.98	127.0	131.7
SpectraMax M5	0.41	0.98	54.3	101.5

Table 2. Lower limits of quantitation and standard curve EC_{50} values obtained with SpectraMax readers.

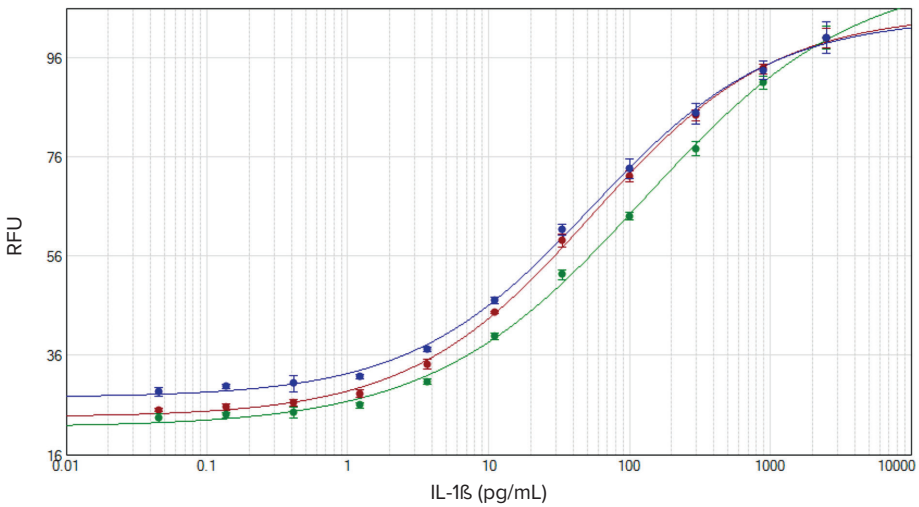

Figure 2. IL-1ß standard curves. The 4-parameter curve fit in SoftMax Pro Software was used to plot data generated with SpectraMax iD3 (red), SpectraMax i3x (green), and SpectraMax M5 (blue) readers.

Assay procedure

Human IL-ß Standard and Human TNF-α Standard were each reconstituted with 0.5 mL of deionized water to prepare standard concentrates. Standards were serially diluted 3-fold (IL-1ß) or 4-fold (TNF-α) in reagent diluent (PBS + 1% BSA) to make a standard curve for each. For IL-1ß the standard concentrations ranged from 2500 pg/mL down to 0.046 pg/mL, and for TNF-α they ranged from 1000 pg/mL down to 0.244 pg/mL.

For the IL-1ß assay, 50 µL of Sword Diluent for IL-1ß was added to each well, followed by 50 µL of standard per well. For the TNF-α assay, 100 µL of Sword Diluent for TNF-α was added to each well, followed by 100 µL of standard. Plates were sealed and incubated for two hours at room temperature on a shaker. Plates were then aspirated and washed as described in the plate preparation section above.

Human IL-1ß and TNF-α Detection Antibodies were reconstituted with 1 mL of calibrator diluent (10% BSA in PBS) or 1 mL reagent diluent (PBS + 1% BSA), respectively. Each detection antibody was then diluted to a working concentration as indicated on its lot-specific Certificate of Analysis from R&D Systems. 100 µL of diluted detection antibody was added to wells, and plates were sealed and incubated at room temperature for two hours on a shaker away from direct light. Aspiration and wash steps were repeated as described previously.

Sword ELISA HRP Conjugate was diluted 1:10,000 in reagent diluent, and 100 µL was added to each well. Plates were sealed and incubated for 20 minutes at room temperature on a shaker away from direct light. Aspiration and wash steps were repeated as described previously.

150 µL of Sword Booster solution was added to each well, and plates were incubated for 15 minutes at room temperature in the dark without shaking. 150 µL of Sword Development solution was then added to each well, and plates were incubated for 30 minutes at room temperature in the dark, with no lid or seal, and caution was taken to avoid agitating the full wells and spilling their contents.

Plates were read on SpectraMax readers using the settings shown in Table 1.

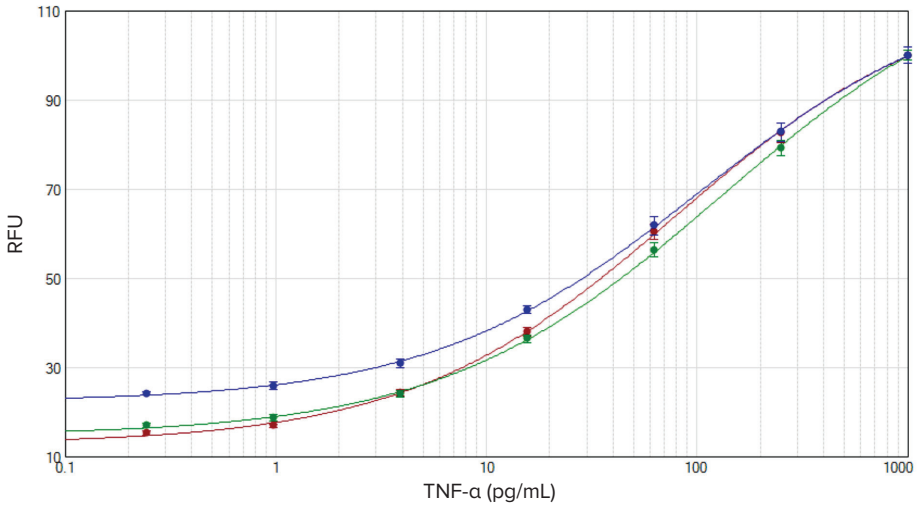

Figure 3. TNF-α standard curves. The 4-parameter curve fit in SoftMax Pro Software was used to plot data generated with SpectraMax iD3 (red), SpectraMax i3x (green), and SpectraMax M5 (blue) readers.

Results

Standard curves were generated by plotting the mean RFU values for the standards vs. the concentration of the standards. In SoftMax Pro Software, data were fit to a four-parameter logistic curve as recommended by Sword Diagnostics.

Assay performance was evaluated using the recommended parameters of limit of detection (LOD) and low limit of quantitation (LLOQ). LOD as defined by Sword Diagnostics is the lowest concentration of standard with signal greater than the sum of the mean zero standard and two times the standard deviation of the zero standard values. LLOQ is defined as the lowest concentration of standard at or above the LOD, with back-calculated accuracy of 80% to 120% and CV of 25% or less.

All three SpectraMax readers that were tested met the criteria indicated in each product insert for LLOQ. An LLOQ of 0.42 pg/mL is typical for the IL-1ß kit, while 0.98 pg/mL is indicated for the TNF-α kit. SpectraMax readers yielded LLOQ of 0.41 pg/mL for IL-1ß and 0.98 pg/mL for TNF-α (Table 2).

Standard curves for IL-1ß and TNF-α were plotted in SoftMax Pro Software (Figures 2 and 3). EC_{50} values were comparable among all three readers for each assay (Table 2).

Conclusion

All three SpectraMax readers tested met the criteria for sensitivity outlined for the Sword ELISA Boosters. This enhanced assay enables quantitation of 0.41 pg/mL of IL-1ß, compared to the 3.9 pg/mL sensitivity attributed to R&D Systems DuoSet for Human IL-1ß with TMB substrate. TNF-α can be quantitated to 0.91 pg/mL, compared to 15.6 pg/mL for R&D Systems DuoSet for Human TNF-α. This 9- to 17-fold increase in sensitivity allows you to detect lower levels of these cytokines and advance your understanding of the diseases associated with these mediators of inflammation.

Molecular Devices readers validated for Sword ELISA Booster detection include:

- SpectraMax® Paradigm® Multi-Mode Microplate Reader
- SpectraMax® i3x Multi-Mode Microplate Reader
- SpectraMax® iD3/iD5 Multi-Mode Microplate Readers
- SpectraMax® M3/M4/M5/M5e Multi-Mode Microplate Readers
- FilterMax™ F5 Multi-Mode Microplate Reader

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