Bio-Plex® suspension array system

tech note 3013

Versatility of the Bio-Plex System: Application to the Detection of Bacterial Protein Toxins

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Introduction

The Bio-Plex suspension array system allows simultaneous detection and quantitation of up to 100 molecules in a single well of a microplate with sensitivity and dynamic range better than that of traditional ELISA methods. Bio-Plex assays are available for the detection and quantitation of cytokines and a series of phosphoproteins. However, when assays are not yet commercially available, they can be designed in-house using capture and detection antibodies raised against purified antigens and ligands.

The Bio-Plex amine coupling kit and COOH beads were used to demonstrate the feasibility of an immunoassay designed in our laboratory for detection of two staphylococcal protein toxins, epidermolysins A (ETA) and B (ETB) (Gravet et al. 2001). Only a few *Staphylococcus aureus* strains produce these toxins, which are associated with bullous impetigo and staphylococcal scalded skin syndrome of neonates and young infants. These toxins induce desquamation for variable periods, with a risk of secondary infections, and may be the source of epidemics in pediatric services (Prévost et al. 2003). We previously purified ETA and ETB and obtained specific rabbit polyclonal affinity-purified antibodies (Cavarelli et al. 1997). While these toxins have 56% sequence identity, the corresponding antibodies give very specific results when tested by radial gel immunoprecipitation (limit = 0.5–2 µg/ml) or immunoblotting (limit = 2–5 ng/ml).

Methods

Covalent Binding of ETA and ETB to COOH Beads

Rabbit polyclonal affinity-purified anti-ETA and anti-ETB antibodies were coupled to Bio-Plex COOH beads 24 and 43, respectively. The coupling reactions used 9 µg of purified antibodies and 1.25 x 10^6 beads as recommended in the Bio-Plex amine coupling kit instruction manual.

Labeling of Anti-ETA and -ETB With Biotin

Each purified antibody (2 mg) was labeled with biotin for 1 hr at 25°C in the presence of a 1/15 molar excess of sulfosuccinimidyl-6-(biotinamido)hexanoate (Uptima).

System Evaluation

Qualitative labeling of beads and soluble antibodies was confirmed with a Deltascan TM4000 spectrofluorometer (Photon Technology Inc.) after mixing and incubating the coupled beads with antigens for 1 hr at 20°C, then with soluble and labeled antibodies for 40 min at 20°C, and then with phycoerythrin for 10 min at 20°C. All steps were performed with shaking in the dark, washing between each step with 100 µl of PBS + 0.05% (v/v) Triton X-100 (PBST).

Validation and Calibration

Optical alignment and reporter channel performance were optimized according to previously published procedures (Etienne et al. 2001). Recognition and classification efficiency of beads of defined color regions as well as fluidics and reading efficiencies were verified daily.

Assay

After prewetting the wells of a porous, flat-bottom microplate with PBST, 2,500 coupled beads were added per working well and then washed by vacuum filtration with PBST. Coupled beads were incubated with samples containing antigens for 1 hr at 20°C in the dark with agitation, washed 2 times with PBST, incubated with 0.8 µg of detection antibody in 50 µl PBST for 45 min at 20°C in the dark with agitation, and washed 3 times with PBST. The beads were incubated with 100 ng of phycoerythrin in 50 µl per well for 10 min at 20°C in the dark with agitation, followed by 2 washes with PBST. The beads were resuspended and analyzed using the Bio-Plex suspension array system.

Results

Sensitivity of Assays

After optimization of the number of beads, the amount of soluble antibody bound per assay, and the number of beads to be detected per color region to record a significant result, the standard curves generated (see figure) showed high linearity between fluorescence and antigen concentration. The range was from 10 mean fluorescence intensity (MFI) units (~2 pg/ml of ETA or ETB) to 23,000 units (~5,000 pg/ml of ETA or ETB). An average background of 10 units was recorded (results were not reproducible below 10 units). Therefore, sensitivity appeared higher than most ELISA methods and the curve was linear for about 3 orders of magnitude; higher concentration samples needed further dilution.

Specificity of Assays

Each possible combination for true or false positive sandwich assays was tested in the presence of high antigen concentrations up to 80,000 pg/ml (Table 1). Only one heterogeneous combination gave a signal at the higher concentration (80,000 pg/ml) considered equivalent to 3–5 pg/ml of a true combination, while other dilutions gave insignificant fluorescence. This false positive result was judged insignificant, based on later results on dilutions of the tested samples.

Table 1. Specificity of assays.

Combination	Range of Heterogeneous Antigen Concentrations, pg/ml	MFI
Anti-ETA beads + ETB + anti-ETA	1–80,000	ND*
Anti-ETA beads + ETB + anti-ETB	1–80,000	ND
Anti-ETA beads + ETA + anti-ETB	1–80,000	ND
Anti-ETB beads + ETA + anti-ETB	1–80,000	ND
Anti-ETB beads + ETA + anti-ETA	1–80,000	130**
Anti-ETB beads + ETB + anti-ETA	1–80,000	ND

* Not detectable.
**130 units = 80 ng, 3–5 pg/ml of homogeneous antigen.

Reproducibility of Assays

Results were highly reproducible from one well to another — whether simultaneously treated or not — and from one titration plate to another from day to day. Furthermore, from a standard titration curve, dilutions of antigens provided results with a correlation coefficient (r^2) of 0.995. Variations were only observed between different lots of coupled beads. Tested for both toxins in 9% (w/v) NaCl, TY medium, or Mueller-Hinton medium, the variations were:

Concentration of toxins, pg/ml	CV, %
2,000–16,000	<2
60–2,000	<8
1–60	8–15

Comparison of Toxin Detection Methods in a Collection of 85 Strains of *S. aureus*

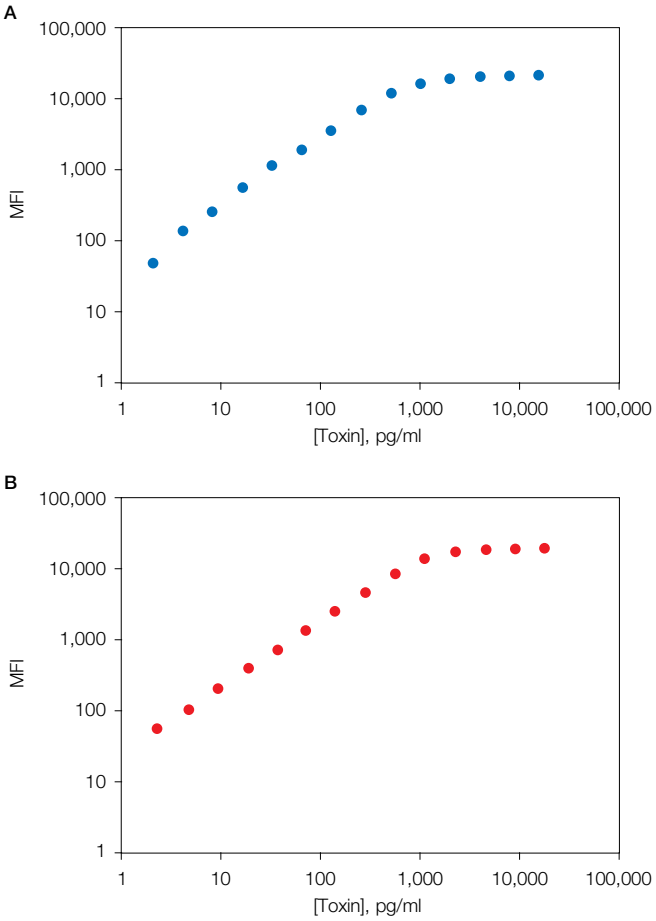
Strains were grown overnight in 0.6 ml of TY medium at 37°C under 10% CO_2. Culture supernatants were used for the multiplex assay at 1/10,000 and 1/50,000 dilutions in TY medium (Table 2). Bio-Plex analysis showed an average of >800 µg/ml ETA produced, and an average of 12–800 µg/ml ETB.

Table 2. Evaluation study.

Tests	Classified Strains			
	ETA+/ETB+	ETA–/ETB+	ETA+/ETB–	ETA–/ETB–
Radial immunoprecipitation	26	9	25	25
Bio-Plex system	27	9	24	25
PCR	27	9	24	25

Discussion

Current PCR methods used for bacterial detection, while highly sensitive, do not provide information about viability of the microorganisms and the expression levels of different bacterial proteins. Direct detection of these proteins, therefore, may be required, especially when testing for protein toxins — the molecules that can be present far from the secreting cells, persist after death of the cells, be present because of external and contaminating intervention, or arise from expression and function of multiple genes. In all of these cases, the Bio-Plex system, with its high sensitivity and its multiplexing advantage, may offer an alternative to track the presence of given compounds directly in the specimen, among several other possibilities. The versatility of the system enables this array



Standard curves for toxins. A, ETA; **B,** ETB.

technology to be applied in different diagnostic areas, including specialized microbiology, serology, and allergy diagnosis. We demonstrated the feasibility of a rapid detection test for staphylococcal ETA and ETB, using affinity-purified antibodies coupled to Bio-Plex COOH beads and purified protein toxins. This test showed high specificity and a detection limit of ~2 pg/ml for each toxin, with very good internal reproducibility.

Assays were easily calibrated and completed in <4 hr, and they showed 100% correlation with a PCR method. The assays demonstrated variable levels of ETB production that other detection techniques would have interpreted as false negative results. The method represents a promising approach for the rapid quantitation of toxic compounds, antigens, or antibodies, whose detection and quantitation represent important clinical information.

References

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