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New SA-PE Conjugates Improve Critical Assay Parameters in Sandwich Immunoassays Performed Using Luminex® xMAP® Technology

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Abstract

In Luminex® xMAP® assay development, the conjugated reporter dye streptavidin-phycoerythrin (SA-PE) can greatly influence such critical assay parameters as the signal strength, limit of detection, background and reproducibility of an assay. We present as an example a pair of antibody-coupled microspheres which showed significant differences in these parameters. Several different types of SA-PE were evaluated and compared.

Introduction

Assay development is a process by which the various components of the assay are optimized with respect to each other to maximize performance. Selecting the optimal antibody pair is a critical part of sandwich immunoassay development. Considerable effort may be necessary to minimize non-specific binding, which can lead to high background (false signals) and poor sensitivity. Also important, but more easily overlooked, is the potential for nonspecific interaction with the reporter conjugate, SA-PE. SA-PEs prepared in a variety of different ways have different properties which can contribute significantly to improved assay characteristics, whether the main objective is highest sensitivity, lowest background or a balance of the two.

Increasingly, assay developers are looking for the highest achievable signals and low limits of detection. New SA-PEs have been developed to meet these objectives, but as a consequence of the manufacturing processes they can have an increased potential for nonspecific binding. Nevertheless, by screening different SA-PEs it is possible to achieve low background and high signal for problematic antibody pairs.

Two assays provided by Radix BioSolutions® were tested (Assay 1 and Assay 2). Assay 1 generated higher than normal background; background counts ranged from one hundred counts to more than ten thousand counts, depending on the SA-PE tested. Assay 2 is more typical, exhibiting low non-specific binding of SA-PE. Twelve lots of PhycoLink® SA-PE, which were intentionally manufactured to be different (*i.e.* molar ratio, degree of conjugation, *etc.*), were compared for their signal and background. Selected SA-PE lots were then tested to determine their effects on limits of detection in the two assays.

Methods

Using a protocol provided by Radix BioSolutions (microspheres 58 and 51 plus their respective antigens and detection antibodies in a duplex sandwich immunoassay format), different SA-PEs were tested in Assay 1 and Assay 2 at varying antigen concentrations using a Bio-Plex™ 200 instrument running the Bio-Plex Manager™ Software v4.1. First, the antibody-coupled microsphere mixture was incubated with the multiplex antigen dilution series (or buffer for background measurements). The microspheres were washed and then incubated with the multiplexed biotinylated detection antibodies. Then the microspheres were washed again and incubated with the specified SA-PE. Finally, the microspheres were washed a final time, resuspended in assay buffer and analyzed (Tables 1 and 2).

The tested SA-PEs included some samples which were chosen because they generated the highest signal in two human cytokine assays, and other samples which were chosen because they produced low backgrounds (data not shown). One sample from a competitor was included as well.

Six of the SA-PEs were re-tested in triplicate with another dilution series of antigen, ranging from 320 pg/ml to 0.32 pg/ml (Assay 1), and from 610 pg/ml to 0.61 pg/ml (Assay 2) in a half-log dilution series, plus multiple no-antigen blanks. Measurements were made at both high and low PMT settings (Table 3). The limit of detection was determined from these measurements as the lowest antigen concentration for which the average MFI was at least 3 standard deviations above the mean of the blank samples.

Results

Table 1 Variation in Background Between Different SA-PEs

SA-PE Product Code	Lot Number	Background MFI Assay 1	Background MFI Assay 2
PJ31S	841 063	8320	9
PJ31S	841 072	5147	10
PJRS20	DH26 005	339	9
PJRS20	DH26 006	1082	10
PJRS30	DH23 002	640	10
PJRS30	DH23 008	351	8
PJRS34	DH23 013	44	9
PJS25	DH28 002	15	8
PJRS50	DH24 001	12	8
PJRS27	DH29 003	11	9
PJRS70	DH25 001	10	8
PJ33S	DE29 159	20	7

Table 2a Sensitivity Differences Between Different SA-PEs

Assay 1 — High PMT Setting (SA-PE Product Code)

Antigen (pg/ml)	PJ31S	PJ70S	PJRS34	Competitor
10833	26383	28103	27419	24064
3611	24693	26731	26838	14740
1204	23844	14736	23733	2470
401	7845	2019	5513	364
134	2222	377	605	85
45	1249	106	149	47
15	1103	65	88	45
0	927	60	52	43

Assay 1 — Low PMT Setting (SA-PE Product Code)

Antigen (pg/ml)	PJ31S	PJ70S	PJRS34	Competitor
10833	15719	16534	22147	7620
3611	12511	11344	17239	3703
1204	6892	3984	7204	728
401	2135	603	1395	116
134	664	108	179	25
45	338	31	45	13
15	288	18	21	12
0	176	17	15	10

Table 2b Sensitivity Differences Between Different SA-PEs

Assay 2 — High PMT Setting (SA-PE Product Code)

Antigen (pg/ml)	PJ31S	PJ70S	PJRS34	Competitor
20833	25481	27007	26645	18942
6944	23892	24011	24707	6874
2315	15267	9575	13669	1843
772	3973	2255	3535	416
257	730	507	574	109
86	177	167	124	53
29	93	87	78	45
0	49	64	48	45

Assay 2 — Low PMT Setting (SA-PE Product Code)

Antigen (pg/ml)	PJ31S	PJ70S	PJRS34	Competitor
20833	12491	12456	16847	4761
6944	7493	6852	9945	1639
2315	3651	2336	3640	489
772	953	572	1043	118
257	221	151	188	29
86	61	54	56	14
29	36	26	25	12
0	20	17	11	11

Table 3a Determining Limit of Detectability

Example: PJRS34 Lot DH23 013 in Assay 1 at High PMT Setting

Antigen (pg/ml)	Number of Replicates	MFI Average	S.D.	Mean + 3 x St. Dev.
320	3	1702		
100	3	236		
32	3	91		
10	3	67		L.O.D.
3.2	3	54		
1.0	3	52		
0.32	3	52		
0	11	50	2.1	56.4

Table 3b Limit of Detectability (pg/ml)

Product Code	Lot Number	High Assay 1	Low Assay 1	High Assay 2	Low Assay 2
PJ31S	**841 072**	320	N.D.	61	19
PJRS20	**DH26 005**	32	32	61	19
PJRS30	**DH23 008**	32	32	**19**	**6.1**
PJRS34	**DH23 013**	**10**	**10**	**19**	19
PJSR25	**DH28 002**	32	32	**19**	19
PJRS27	**DH29 003**	100	32	61	61

Discussion of Results

In previous work developing a multiplex xMAP immunoassay, high background was observed for Assay 1 in the absence of either antigen or detection antibody. Further testing revealed that the high background was caused by the SA-PE. Multiple lots of SA-PE (product code PJ31S) were tested, and all were found to display high background for Assay 1, but not for Assay 2 (nor for other assays in the multiplexed set, which are not shown). In all other respects, PJ31S was the optimal choice at the time because of its high signal strength.

We have developed and tested additional types of SA-PE (product codes PJ70S, PJRS20, PJRS30, PJRS34, PJRS25, PJRS27 and PJ33S) in a continuing effort to obtain high sensitivity and low background in xMAP applications. In this study, we identified candidates with decreased Assay 1 backgrounds (Table 1). These included new SA-PEs (PJRS20, PJRS30 and PJRS34) which have been shown to generate robust signals comparable to PJ31S SA-PE in human cytokine assays (not shown). Of these SA-PEs, the lowest background in Assay 1 was obtained using PJRS34.

In another experiment, several SA-PEs were tested across a broad antigen dilution series (Tables 2a & 2b). The purpose was to characterize the relative fluorescence intensities produced by the SA-PEs at low, medium and high antigen concentrations. Whether the samples were analyzed at the high or the low PMT setting on the Bio-Plex™ 200, a high background was observed with PJ31S in Assay 1 that was not seen with the other SA-PEs. Of the others, PJ70S and PJRS34 show significantly higher signal strength across the entire antigen concentration range. The same differences were found in Assay 2, which shows that the background reduction is achieved for Assay 1 by PJRS34 and PJ70S without sacrificing sensitivity in either assay.

Five of the SA-PEs with either high signal strength and/or low background were then compared more extensively with PJ31S at low antigen concentrations to determine the limits of detectability. For this analysis, we have defined the limit of detectability as >3 standard deviations above the mean background MFI. The antigen-based signal is an average of triplicate measurements, and the background is an average of 11 measurements. Table 3a shows how this is determined for one of the SA-PEs. Table 3b shows the compiled determinations for the six tested SA-PEs in both assays and at both high and low PMT settings.

In each column (which contains data for a given assay and PMT setting), the best result(s) is/are shown in bold. Most of the SA-PEs are fairly similar, except for PJ31S in Assay 1 (which is due to the high background) and PJRS27, which has the lowest signal strength of the six tested SA-PEs. The result with PJRS27 is not surprising, since it was developed for a different application where characteristics other than signal strength were considered more important. Overall, the best performer is PJRS34, with PJRS30 being nearly equivalent. This is consistent with the characteristics of these SA-PEs shown in previous data; they produce high signal strength combined with low background.

Conclusions

SA-PEs made by different processes can greatly affect assay performance and sensitivity; we have observed differences in overall signal, background and limit of detectability. The new SA-PEs described in this study (PJRS34, PJRS30, PJRS20 and PJRS25) not only show greatly reduced background, but the signal in the presence of antigen remained high: overall this resulted in an increase of approximately 1/2 log in sensitivity. Our results clearly show that the specific preparation of SA-PE will affect the performance of an assay. We believe that selecting the optimal SA-PE for a specific immunoassay is best determined empirically, and an optimal assay will only be produced when screening of different SA-PEs is integrated with the screening of capture and detection antibodies during assay development.

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