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Enhanced sensitivity and precision in an enzyme-linked immunosorbent assay with fluorogenic substrates compared with commonly used chromogenic substrates

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Abstract

Quantitative enzyme-linked immunosorbent assay (ELISA) is a widely used tool for analyzing biopharmaceutical and vaccine products. The superior sensitivity of the ELISA format is conferred by signal amplification through the enzymatic oxidation or hydrolysis of substrates to products with enhanced color or fluorescence. The extinction coefficient for a colored product or the quantum yield of a fluorescent product, coupled with the efficiency of the immobilized enzyme, is the determining factor for the sensitivity and precision of a given ELISA. The enhancement of precision and sensitivity using fluorogenic substrates was demonstrated in a direct-binding ELISA in a low-analyte concentration range compared with commonly used chromogenic substrates. The enhancement in precision was demonstrated quantitatively with lower coefficients of variation in measurements of signal intensities, approximately a five- to six-fold enhancement in signal-to-noise ratio at a given analyte concentration with fluorogenic substrates. Similarly, the amplitude of the enhancement in sensitivity, as reflected by relative limits of detection or quantitation, is approximately two- to five-fold when compared with commonly used chromogenic substrates. Additional advantages of a fluorescence-based ELISA format include the continuous monitoring of initial rates of enzymatic reactions, the measurement of fluorescence changes in the presence of particulate materials, the absence of a quench step, and a larger quantifiable analyte range.

Keywords: ELISA; Enzyme substrate; Fluorescence; Fluorogenic substrate; Level of detection; Level of quantitation; Z-factor; Immuno assay; Affinity constant determination; Sensitivity; Precision

Since the development of the first radioimmunoassay (RIA)[1] in 1959, there has been a constant search for more sensitive, robust, and reproducible immunoassays for life science, research and development, and clinical diagnostics. During recent years, because of progress in genomics and proteomics, more sensitive and robust binding assays are being sought for high-throughput screening and microarray applications. The enzyme-linked immunosorbent assay (ELISA) is replacing the RIA format as the most widely used immunochemical

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[1] *Abbreviations used:* RIA, radioimmunoassay; ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidase; AP, alkaline phosphatase; HBsAg, hepatitis B surface antigen; mAb, monoclonal antibody; S/N ratio, signal-to-noise ratio; LOD, limit of detection; LOQ, limit of quantitation; OPD, *o*-phenylenediamine; 4-MUP, 4-methylumbelliferyl phosphate; TBS, Tris-buffered saline; BSA, bovine serum albumin; %CV, relative coefficient of variation; SD, standard deviation; ICH, International Conference of Harmonization; TMB, 3,3′,5,5′-tetramethyl-benzidine; 4-MU, 4-methyl umbelliferone.

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tool in pharmaceutical and biological research and development as well as in clinical settings. High throughput, ease of use, and the absence of radioactive reagents make the ELISA format the method of choice for many applications.

The sensitivity and precision of an ELISA are determined by several factors. Affinities of the primary and secondary antibodies are generally the deciding factors for precision and sensitivity of an ELISA. In addition, the amount of enzyme immobilized, the efficiency of the immobilized enzyme, and the subsequent intensity changes in the absorbance or fluorescence incurred by enzymatic hydrolysis or oxidation also dictate the assay sensitivity and precision in an ELISA. Three highly efficient and commonly used enzymes for ELISAs are horseradish peroxidase (HRP), alkaline phosphatase (AP), and β-galactosidase [1–5]. With an increasing number of fluorogenic substrates available for these enzymes, the sensitivity of a fluorescence-based ELISA can be further enhanced with different enzyme/substrate combinations. In general, a larger dynamic range for the analyte quantitation and enhanced sensitivity/precision are the two advantages of the fluorescence-based detection format over the absorbance-based detection format.

In this article, we report the evaluation and comparison of absorbance- and fluorescence-based ELISAs with respect to sensitivity and precision. To guide enzyme/substrate selection, and to demonstrate the advantages of the fluorescence-based ELISA format, several fluorogenic substrates were evaluated using a direct-binding ELISA for quantitation of a specific mouse anti-hepatitis B surface antigen (HBsAg) monoclonal antibody (mAb), B4 (referred to as the "analyte"). The precision of the assays with different substrates was analyzed with respect to the coefficients of variation in signal intensity values, signal-to-noise (S/N) ratios, and screening window coefficients (Z factors) at different analyte concentrations. The sensitivity for the ELISA was defined by the limit of detection (LOD) or the limit of quantitation (LOQ) at the lower end of the quantifiable analyte concentration range. The advantages of fluorogenic substrates in the ELISA format for quantitative analyses are illustrated by the larger analyte concentration and signal intensity range, markedly improved precision, and two- to five-fold enhancement in sensitivity.

Materials and methods

Materials

In this study, 96-well white FluoroNUNC MaxiSorp and clear Immunolon 4 microtiter plates were used for fluorescence- and absorbance-based ELISAs, respectively. Purified recombinant HBsAg (overexpressed in yeast) was at least 95% pure and stored at 0.1–0.3 mg/ml in phosphate-buffered saline [6]. The concentration of the purified HBsAg protein was determined with the Lowry method [7]. An anti-HBsAg-specific mAb, B4 (IgG_1, λ, Merck), was purified (⩾95% pure) from the hybridoma cell culture supernatant with a protein A column. The mAb concentration was calculated from the absorbance intensity at 280 nm, assuming an absorbance value of 1.4 (with 1 cm path length) for 1.0 mg/ml IgG [8]. The secondary Ab conjugates (rabbit anti-mouse IgG [heavy and light chain or H + L] AP conjugate and rabbit anti-mouse IgG [H + L] HRP conjugate) were purchased from Pierce (Rockford, IL, USA). The 96-channel automatic ELx-405R plate washer was obtained from Bio-Tek (Vinooski, VT, USA). Multichannel pipetting and serial dilutions were performed on a BioMek 2000 liquid handling station with an MP200 tool (Beckman Coulter, Fullerton, CA, USA).

Enzyme substrates

Chromogenic and fluorogenic substrates for AP or HRP were obtained from the vendors listed in Table 1.

Table 1
Chemical names and properties of chromogenic and fluorogenic substrates tested in the same ELISA for comparative studies[a]

Abbreviation	Chemical name	Trade name	Source	Enzyme	Wavelength (nm)	References
Fluorogenic substrates						
ADHP[a]	10-Acetyl-3,7-dihydroxyphenoxazine	SuperRed	ViroLabs (Chantilly, VA)	HRP	Ex: 531, Em: 595	[9–11]
HPPA	3-(*p*-Hydroxyphenyl)propionic acid	QuantaBlu	Pierce (Rockford, IL)	HRP	Ex: 325, Em: 404	[4,5,12]
BBTP	2′-[2-Benzothiazoyl]-6′-hydroxy-benzothiazole phosphate	AttoPhos	Promega (Madison, WI)	AP	Ex: 435, Em: 575	[3,13,14]
4-MUP	4-Methylumbelliferyl phosphate		Molecular Probes (Eugene, OR)	AP	Ex: 360, Em: 450	
Chromogenic substrates						
TMB	3,3′,5,5′-Tetramethyl-benzidine		Sigma (St. Louis, MO)	HRP	Abs. max: 450	
OPD	*o*-Phenylenediamine		Pierce (Rockford, IL)	HRP	Abs. max: 490	

Note. QuantaBlu, Attophos, TMB, and OPD need a quench/stop step prior to the plate reading; whereas 4-MUP and ADHP do not require a quench step.

[a] The chemical compound ADHP is marketed by Molecular Probes under the trade name Amplex Red. The formulated liquid substrate of ADHP is marketed by ViroLabs with the trade name of SuperRed.