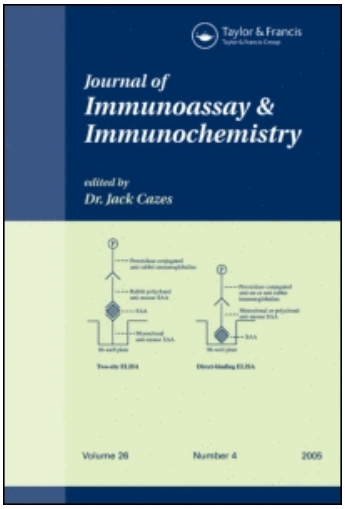

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3-p-HYDROXYPHENYLPROPIONIC ACID - A SENSITIVE FLUOROGENIC SUBSTRATE FOR AUTOMATED FLUOROMETRIC ENZYME IMMUNOASSAYS

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ABSTRACT

The application of 3-p-hydroxyphenylpropionic acid (HPPA), a fluorogenic substrate of horseradish peroxidase (HRP) to an automated microplate fluorometric enzyme immunoassay is described. Fluorescence intensity of the end product was highly dependent on the pH of the buffer and on the concentrations of the substrate mixture ingredients.
The determination of human thyrotropin (TSH) and recombinant hepatitis B surface antigen (rHBsAg) were performed using a fluorometric enzyme immunoassay (FEIA) with HPPA as the substrate, and a colorimetric one with tetramethylbenzidine (TMB) as the chromogenic substrate. The sensitivity of both types of assays proved comparable.
The distinct advantage of a fluorometric assay is the possibility to perform a quantitative detection of analyte over a very wide dynamic range.
Clinical evaluation of both assays showed good correlation between the FEIA and conventional methods.

(KEY WORDS: horseradish peroxidase, 3-p-hydroxyphenylpropionic acid, fluorometric enzyme immunoassay).

INTRODUCTION

Recent developments in enzyme immunoassay technology have greatly emphasized the advantages of the fluorometric detection method. Its sensitivity and measuring range are significantly better than those of absorptiometric detection, which has made it the method of choice in several novel immunoassay systems. Sensitive fluorogenic substrates exist for all enzymes commonly used in enzyme immunoassays (1).

The first comprehensive publication concerning fluorogenic substrates for horse radish peroxidase (HRP) was published in 1980 by K. Zaitsu and Y. Ohkura (2). The authors tested a wide variety of hydrogen donating substances, among which 3-p-hydroxyphenylpropionic acid (HPPA) proved to be the most sensitive. Further studies performed by Ishikawa (1) showed that HPPA was 5 to 50 fold (depending on assay time) more sensitive than o-phenylenediamine - a chromogenic substrate commonly used in HRP-labelled enzyme immunoassays (EIA).

Later HPPA was successfully used in sandwich fluorometric EIAs (FEIAs) for the detection of different substances such as human insulin, alpha-fetoprotein and human chorionic gonadotropin (3-6). However, the FEIAs

were performed in test tubes and the measurement took place in single cuvette spectrofluorometers which did not allow automation of the procedure.

The dynamic range of photometric EIAs is restricted by photometers, which maximally may read absorbances up to 2 to 4 optical density units (OD). FEIAs performed with fluorogenic substrates have a considerable advantage because of the wide dynamic range of the instrument, which allows detection of very low as well as very high concentrations of analyte.

The aim of our study was to adapt HPPA as a substrate for measurements in microplates, using small volumes and read with an automatic microplate fluorometric reader, to find out the optimum conditions for accurate, rapid and sensitive microtitre volume FEIAs, and to compare HPPA with TMB - a sensitive chromogenic substrate which is presently widely used in HRP-labelled EIAs.

MATERIALS AND METHODS

Reagents

HPPA was purchased from Sigma Chemical Co. (St. Louis, USA), and HRP from Boehringer (Mannheim, FRG).

Hydrogen peroxide (Perhydrol) as well as all salts for buffers were purchased from Merck (Darmstadt, FRG). Human thyrotropin was obtained from Sigma. Horse Serum was produced by Seralab (Crawley Down, UK) and bovine serum albumin (BSA) was obtained from ICN Biomedicals (Irvine, USA) or from Sigma. Monoclonal antibody against TSH beta-subunit was purchased from OEM Concepts (Toms River, USA) and antibody against whole TSH molecule was produced using reported methods (7). HBsAg EIA kit was produced by Labsystems (Helsinki, Finland). rHBsAg (subtype *ay*) was expressed in a Vero-hepatocyte hybrid cell-line by Labsystems.

Specimens

Human serum samples with known TSH activities measured by a commercial IRMA test (Behring, Marburg, FRG) (n=55) were obtained from the Aurora City Hospital, Helsinki. Out of the fifty-five patient samples 19 had TSH activity below or equal to 0.2 mIU/l, 18 had TSH activity ranging from 0.2 to 5.0 mIU/l and 18 patients had TSH activity higher or equal to 5.0 mIU/l.

Human sera positive for HBsAg confirmed by a commercial EIA test (Abbott, Illinois, USA) (n=25) were obtained from the Department of Virology, Helsinki

University and sera negative for HBsAg (n=17) were obtained from the Finnish Blood Transfusion Centre, Helsinki.

Equipment

The microplate fluorometer (FLUOROSKAN 2), microplate photometer (MULTISKAN PLUS), as well as transparent and black microplate strips were manufactured by Labsystems.

Determination of Optimal HPPA/H_2O_2 Concentrations for HRP Enzyme Reaction

HRP stock solution containing 9.4 µg/l of HRP and 5 g/l of BSA in phosphate buffered saline (PBS) was prepared. 50 µl of HRP solution was pipetted into black microstrip wells, and 165 µl of HPPA (at concentrations of 10, 7.5, 5, 3.8 and 2.5 g/l in 0.1 M hydroxymethylaminomethane (Tris), pH 7.8) was added. The reaction was started by adding 35 µl of H_2O_2 (at concentrations of 1.2, 0.6, 0.3, 0.15, 0.06, 0.03 and 0.015%). The reaction was allowed to proceed for two minutes at room temperature with agitation, and stopped by adding 100 µl of 1.5 M glycine, pH 10.3. The fluorescence was measured at 405 nm (excitation at 320 nm).

Determination of TSH by EIA/FEIA

Human TSH was determined in serum using a sandwich enzyme immunoassay based on two murine monoclonal antibodies. One antibody was conjugated with HRP as described by Ishikawa et al. (1) and the other antibody was attached to the bottom of black or transparent microstrip wells. TSH standards were prepared in horse serum. In the assay 100 µl of each standard and 100 µl of HRP-conjugate (diluted with 20 mM Hepes buffer, pH 7.0, containing 1 g/l BSA, 150 mM sodium chloride and 0.1% Tween 20), were pipetted into precoated wells and incubated for 90 minutes at room temperature with agitation. The wells were washed four times with PBS containing 0.1% Tween 20. The substrate reaction was performed using either TMB or HPPA. In the chromogenic method, 200 µl of TMB substrate solution from the Labsystems HBsAg EIA kit was added into each transparent well and incubated for 30 min at room temperature with agitation. The reaction was stopped by adding 100 µl of 2 M sulphuric acid and the absorbances were measured at 450 nm.

The HPPA reaction was performed as follows: five aliquots of HPPA solution (7.5 g/l in 0.1 M Tris buffer, pH 7.8; stability > 2 months) were mixed with one

aliquot of fresh 0.06% H_2O_2 immediately before use. 200 µl of the substrate solution was added into each black microstrip well, followed by incubation in a shaker for one hour at room temperature. The reaction was stopped with 100 µl of 1.5 M glycine buffer (pH 10.3). The fluorescence was measured at 405 nm (excitation at 320 nm).

Determination of HBsAg by EIA/FEIA

HBsAg EIA was performed according to the manufacturer's kit instruction, or alternatively by replacing TMB with HPPA as described above for TSH, but using 100 µl of the substrate mixture and 50 µl of stopping solution as specified in the kit.

Determination of the Sensitivities of TSH and HBsAg Assays

1. Comparison of TSH EIA vs. FEIA sensitivities was performed as follows: standards containing 0, 0.01, 0.05, 0.1, 0,15 and 0.2 mIU/l TSH were assayed in parallel 8 times using TMB/HPPA substrates. Theoretical

sensitivity for the fluorometric assay was calculated according to the equation:

$S_t = 2\ SD_{F0} \times A_{st} / (F_{st} - F_0)$, where

S_t - theoretical sensitivity,

SD_{F0} - standard deviation of the fluorescence of the zero standard,

F_{st} - mean fluorescence of the lowest standard giving coefficient of variation less than 10%,

F_0 - mean fluorescence of the zero standard.

A_{st} - concentration of the standard giving F_{st}.

Theoretical sensitivity for the absorptiometric assay was calculated similarly by replacing the fluorescence values with absorbances.

2. Comparison of HBsAg EIA vs. FEIA sensitivities was performed as follows: recombinant HBsAg was dissolved in PBS with 10 g/l BSA at concentrations of 40, 20, 10, 5, 2.5, 1.25, 0.6, 0.3 and 0.15 U/ml, calibrated against the Reference Preparation of Paul Ehrlich Institute (Frankfurt/Main, FRG)) and assayed with Labsystems commercial HBsAg EIA kit. A cut-off point for the photometric assay was calculated as the mean OD of the three negative controls (provided in the kit) plus 0.1 OD (which was equal to double the OD of a single negative control). For the fluorometric assay the cut-off was calculated similarly as the mean fluorescence

of the three negative controls plus 60 fluorescence units, corresponding to the double fluorescence of a single negative control.

RESULTS

1. Determination of the pH Optimum for the HPPA Substrate Reaction

The intensity of the substrate reaction was estimated in the TSH FEIA using 5 g/l HPPA in the following buffers: 0.1 M sodium acetate pH 4.5, 0.1 M sodium phosphate pH 6.1, 0.1 M sodium phosphate pH 6.8, 0.1 M Tris pH 7.5, 0.1 M Tris pH 8.6, and 0.1 M sodium carbonate pH 9.2. 0.03% H_2O_2 was used as the second component in the substrate mixture containing five aliquots of HPPA and one aliquot of H_2O_2. A wide pH range (6.8-8.6) for the substrate reaction was observed. The serum containing no TSH gave elevated fluorescence values at higher pH's, with the net fluorescence highest at pH 7.8.

2. Optimization of the Substrate Concentrations and Buffers

The concentrations of the HRP substrate mixture components were optimized using the HRP enzyme reaction

as described in Materials and Methods. Concentrations of HPPA vs. H_2O_2 equal to 3.8 g/l and 0.015%, respectively, gave the highest fluorescence. The dependence of fluorescence intensity on HPPA and H_2O_2 concentrations is shown in Figure 1.

A similar experiment was performed using TSH FEIA instead of direct HRP reaction, and it was observed that optimum substrate concentrations were considerably higher in FEIA. The highest response in TSH FEIA was obtained using 7.5 g/l HPPA and 0.06% H_2O_2 (data not shown).

3. Fluorescence Enhancement by the Stopping Solution. Fluorescence Stability.

Termination of the enzymatic reaction by addition of alkaline solution resulted in a considerable enhancement of the fluorescence intensity. Maximum enhancement was obtained with 1.5 M glycine pH 10.3, while 50 mg/l sodium sulfite (pH 9.0) and 3 M sodium hydroxide were less efficient. Glycine addition resulted in almost 5-fold increase in fluorescence at lower TSH concentrations; at concentrations higher than 80 mIU/l the enhancement decreased gradually. The fluorescent end product was stable enough to allow fluorescence mesurement after overnight storage when necessary.

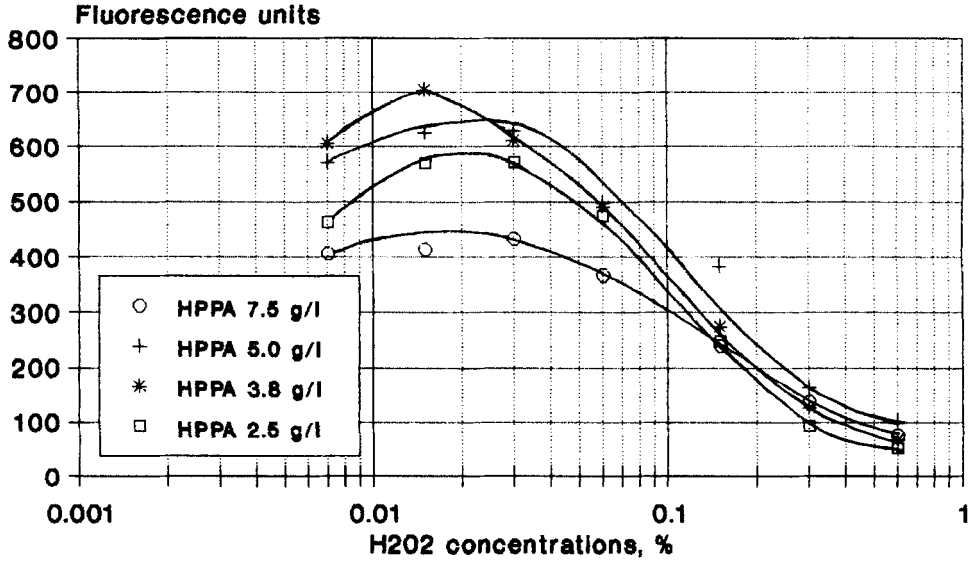


FIGURE 1: Optimization of HRP fluorogenic substrate concentrations

4. Time and Temperature of Substrate Incubation

There was no difference in the sensitivity of the TSH FEIA when substrate incubation took place at room temperature or at 30° C. Incubation at 37° C increased the fluorescence of a sample containing no TSH thus actually decreasing the net fluorescence. Formation of the fluorescent end product was linear with respect to time during the one hour incubation (results are not shown).

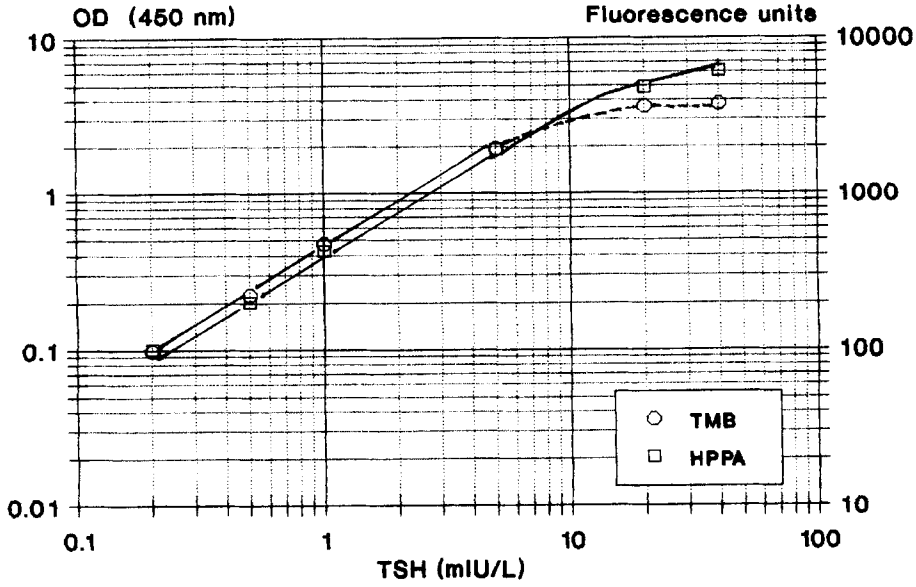
5. Sensitivities of HPPA and TMB as Substrates in TSH Immunoassays and Clinical Performance of TSH FEIA

The sensitivity of the fluorogenic substrate HPPA was compared with the chromogenic TMB in the TSH enzyme immunoassay. As can be seen in the Figure 2A, the two substrates gave parallel standard curves up to the dynamic limit of the photometer.

For the TSH FEIA/EIA comparison the following data were obtained: the zero standard gave 40 $\pm$ 2 fluorescence units or 0.076 $\pm$ 0.006 OD units, and the standard containing 0.05 mIU/l of TSH gave 53 fluorescence units (CV = 4.9%) or 0.107 OD units (CV = 9.3%). Thus the theoretical sensitivity calculated as described in Materials and Methods was 0.014 mIU/l for the fluorometric and 0.0193 mIU/l for the colorimetric assay.

Correlation studies were performed on 55 patient sera which were assayed with EIA and FEIA. The results are seen in the Figure 2B, giving a regression equation of $y = 0.98\ x - 0.19$; (y = FEIA, x = EIA), r = 0.98.

Comparison between the FEIA and the immunoradiometric results gave a regression equation of $y = 1.04\ x - 0.22$; (y = FEIA, x = IRMA), r = 0.98.



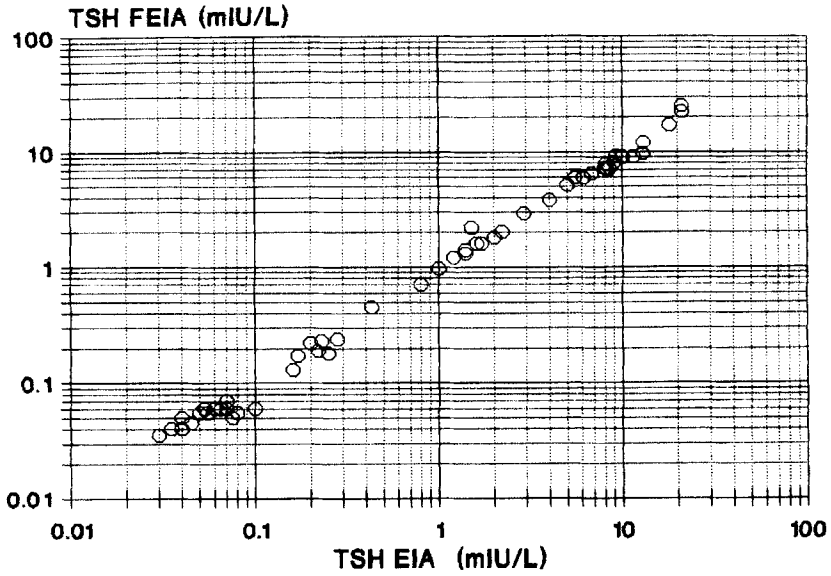
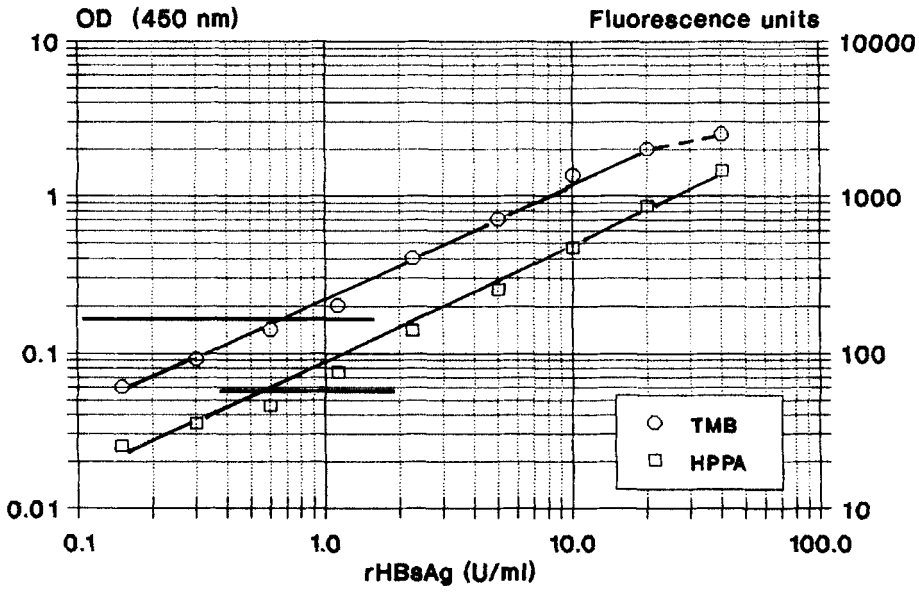


FIGURE 2: A. Calibration curve of TSH EIA vs. FEIA. The dotted part of the EIA curve falls outside the linear measuring range of the photometer.

B. Correlation of TSH EIA and FEIA performed on 55 patient sera.



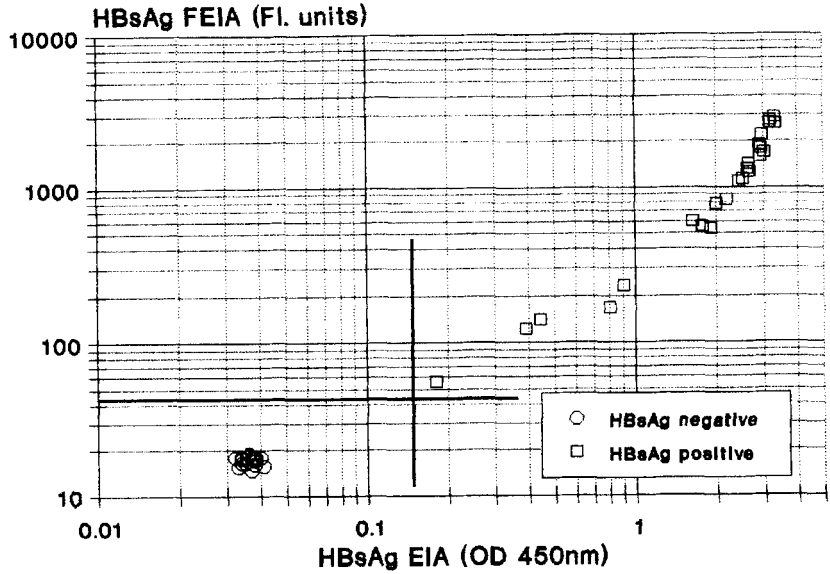


FIGURE 3: A. Calibration curve of rHBsAg EIA vs. FEIA. The dotted part of the EIA curve falls outside the linear measuring range of the photometer.

B. Correlation of HBsAg EIA and FEIA performed on 17 HBsAg negative and 25 HBsAg positive patient sera. Cut-off lines are drawn for the both assays.

6. Sensitivities of HPPA and TMB as Substrates in HBsAg Immunoassays and Clinical Performance of HBsAg FEIA

In HBsAg FEIA/EIA the sensitivity of the two substrates was compared indirectly, by calculating the cut-off points. In both assays concentrations of rHBsAg in the range 0.15 - 0.6 U/ml lie below the cut-off line. HBsAg FEIA/EIA calibration curves are illustrated in the Figure 3A.

Correlation studies were performed with 25 positive and 17 negative patient sera. All negative samples were below cut-off point both in FEIA and in EIA, while all positive samples were definitely over cut-off points. The results are seen in the Figure 3B.

DISCUSSION

The fluorescence intensity resulting from the HPPA/H_2O_2 substrate reaction is influenced by several factors, the most important being the concentrations of both substrates and the pH of the reaction as well as of the stopping solution. We observed (Fig.1) that optimum substrate concentrations in the homogeneous enzyme reaction (19 mM HPPA and 0.74 mM H_2O_2; HPPA/H_2O_2 = 26) were remarkably lower than those re-

quired in microplate format solid phase FEIA (38 mM HPPA and 2.9 mM H_2O_2; HPPA/H_2O_2 = 12.8). This indicates that optimum concentrations are dependent on the assay system used, and should be determined in the specified conditions.

The optimum pH for the enzymatic reaction was 7.8, although the pH dependence was not very steep. On the other hand, the fluorescence intensity of the end product was greatly enhanced when the pH was elevated to 10.3 after the reaction. The fluorescence signal remained relatively stable for several hours after terminating the reaction.

The performance of the optimized detection system was verified in HRP-labelled enzyme immunoassays for TSH and HBsAg. In both cases the results were compared with those obtained using TMB as a chromogenic substrate. Clinical evaluation showed good correlations between FEIA and EIA (Fig. 2B, 3B).

The calculated detection limit of the ultrasensitive TSH with the fluorometric substrate was 0.014 mIU/l, readily enabling reliable detection of values below the reference limit. The upper assay limit was 40 mIU/l, while the corresponding absorptiometric assay only extended to 7 mIU/l due to the limitation of photometer linearity (Fig. 2A).

HBsAg assay showed similar sensitivities with either HPPA or TMB as substrates. The TMB calibration curve reached a plateau at the highest concentration of rHBsAg equal to 40 U/ml, while the HPPA curve remained linear beyond this level (Fig. 3A). Thus, in the HBsAg assay as with TSH, the measuring range was significantly wider with HPPA than with TMB.

The sensitivities of fluorogenic vs. chromogenic substrates have been compared widely by Ishikawa et al. (1), and it is well known that fluorogenic substrates of alkaline phophatase or beta-galactosidase offer sensitivities far beyond their chromogenic counterparts. Also Ishikawa's studies indicated that the fluorogenic HRP substrate, HPPA, was 5 to 50-fold more sensitive than o-phenylenediamine. Remarkably, the present results show that even after careful optimization of the HPPA reaction, the sensitivities of HPPA and TMB as HRP substrates were very similar. The choice between the two will thus depend on factors such as instrumentation and range requirements.

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