HRP Redox Reaction Driven TMB Color Development
Overview

Despite all the interesting and useful advances in enzyme immunoassay (EIA) driven technology, a very significant percentage of the biological sample detection work is still performed using some version of the solid-phase support concept. Two of the more common-place biological sample testing formats are based upon modified solid support surfaces within ELISA plate wells or on multiple composition types of suspension bead surfaces. Much of the EIA signal generation processes involve the use of either alkaline phosphatase (ALP) or horseradish peroxidase (HRP) enzyme activity. Amongst the colorimetric signal generating formats, the HRP-enzyme + 3,3',5,5'-tetramethylbenzidine (TMB)-substrate signal generation tandem is still the preferential option. In contrast, ALP in years past performed much better within a chemiluminescent assay framework than as a colorimetric signal generator in absorbance signal-based assay platforms. Since our focus in this technical discussion is directed at colorimetric signal generation readouts, we will devote our attention to examining the redox relationship that exists between HRP and it two substrates, hydrogen peroxide (H₂O₂) and TMB.

Part 1 of this technical review of key chemical interactions leading to HRP conversion of TMB to its oxidized colored product will include a summary the physical/molecular characteristics of the three key reaction components (HRP, H₂O₂, and TMB) that are responsible for converting the colorless TMB starting substrate into its green oxidation state form. With respect to the HRP assay component, we'll address several important molecular structure features that contribute to its continued utility and versatility. These structural features are the source of this enzyme's excellent stability character and high substrate turnover rate properties. We follow up the HRP enzyme features summary with a brief overview of some past and present chromogenic HRP substrates. We attempt to provide some historical insight into the motivation factors behind the consensus move away from the once popular benzidine-based substrate dyes. It was well documented by several mutagen/carcinogen screening methods that benzidine dyes were confirmed carcinogens. This led to the synthesis of a safe benzidine substrate in the form of TMB. Closing out Part 1, we provide a cursory look at the molecular features of the oxidizing substrate H₂O₂. We briefly address the significance of the peroxide (peroxo) single bond oxygen to oxygen linkage with respect to its influence on the oxidative properties of this molecule.
In Part 2, we take a direct look at the redox reaction mechanism responsible for the conversion of the unoxidized and colorless TMB substrate into a blue-green colored, one electron loss oxidation state product. Special attention is paid to the interactions of the \( \text{H}_2\text{O}_2 \) oxidizing substrate and TMB reducing substrate with respect to their effect on the heme redox site. We define the specific steps within the heme facilitated oxidation and reduction processes leading to the conversion of a colorless TMB starting product into the blue green → yellow respective one and two electron oxidation state end products. We conclude the redox cycle discussion by looking at the redox process from the TMB substrate perspective. An emphasis is placed on defining the TMB oxidation scheme with its multiple oxidation state intermediates. Our TMB oxidation process discussion concludes with a more close-up look at the chemical basis behind the acid-stop conversion of the blue green (one electron loss oxidation state) product into the yellow (two electron loss oxidation state) TMB substrate product.

Part 3 of this review summarizes the chemical relationships existing between HRP and its \( \text{H}_2\text{O}_2 \) and TMB substrates.

Part 1

HRP as a preferred redox enzyme catalyst

Horseradish peroxidase (HRP) has achieved a broad degree of acceptance as a stable and efficient enzyme mediator of chromogenic substrate signal generation. This ascension to prominence was likely the result of several desirable enzyme performance attributes. It is worth mentioning, however, that economic factors associated with an abundant and readily accessible supply of crude HRP extract starting material enhanced the affordability of the final highly purified HRP end-product. Sourced from a common easy to grow plant source, the horseradish plant (Armoracia rusticana), its roots provide an abundant source of the crude HRP enzyme starting material. Crude HRP enzyme extract is subsequently purified into various commercial grades of HRP enzyme product [1]. Abundant supplies of crude HRP starting material combined with the existence of efficient large scale commercial HRP purification processes, made HRP a very affordable option for chromogenic substrate signal generation.

Other contributing factors leading to broad acceptance of HRP as a reliable research and commercial assay kit signal generating enzyme include the stability properties of the enzyme, its catalytic substrate turnover rate, ease of conjugation to immunoglobulin G (IgG) or other protein/hapten, and lastly how much steric hindrance is incurred upon its conjugation to an IgG up/sandwiching antibody. With respect to the above-mentioned favorable attributes criteria, HRP conjugates demonstrate > 18 months shelf-life (2°C – 8°C) when stored in an appropriately formulated conjugate stabilizer buffer. Two such HRP conjugate stabilizer formulations include ImmunoChemistry Technologies’ (ICT’s) Neptune (Catalog # 6347) and Mammalian (Catalog # 6350) HRP conjugate stabilizer products. As an enzyme label for conjugation to analyte-specific IgG, HRP is quite amenable to the conjugation process. It can also be lyophilized in the free molecule or conjugated-to-IgG form when diluted into an appropriate lyophilization medium. Regarding the performance criteria focusing on HRP catalyzed substrate turnover rate, HRP is considered to be a high substrate turnover rate enzyme [2]. With respect to its ease of conjugation to IgG or other proteins, HRP possesses a minimal number of chemically
accessible lysine groups (a total of 4 groups) that allows for a controllable (minimal crosslinking) conjugation process with another protein. Being a glycoprotein, it possesses a relatively large carbohydrate to protein mass ratio. Carbohydrate structure represents between 18% and 22% of the total mass of the HRP enzyme molecule [3, 4]. For several decades, oxidation of the HRP-carbohydrate moieties to form amine-reactive aldehyde functional groups provided a reliable coupling strategy for conjugation of HRP to other primary amino-group bearing proteins [5]. When conjugating HRP to an antigen-specific IgG, it’s relatively small (as enzymes go) molecular weight (42 kDa – 44 kDa), resulted in minimal levels of steric hindrance with respect to antibody binding site function. In contrast, conjugating larger molecular weight signal generation enzymes (e.g. alkaline phosphatase), to a same or different antigen-specific IgG stock will lead to an increased probability of antibody hypervariable binding site interference.

Selection of horseradish peroxidase as a general go-to enzyme mediator of chromogenic or chemiluminescent sourced signal generation in research and commercial venues, was essentially driven by both performance and pricing factors.

**HRP enzyme structure**

Within the multiple isozyme iterations (42 as identified by isoelectric focusing) making up the horseradish peroxidase complement of enzymes, HRP isoenzyme C (HRP-C) is the most abundant. It constitutes approximately 50% of total peroxidase enzyme from horseradish peroxidase [6]. Structurally, the HRP-C isoenzyme is a globular protein comprised of 308 amino acids plus 8 asparagine linked carbohydrate side chains [7, 8]. Early glycosylated molecular weight estimates fell between 42 kDa and 44 kDa depending upon the quantity of carbohydrate content [8]. The 308 amino acids (33,890 Da) plus a heme group (572 Da) plus two calcium ions brings the molecular weight up to the 34,542 Da value [2]. Assuming a 42,000 Da to 44,000 Da glycosylated molecular weight, the carbohydrate content would account for between 18% and 22% of the molecular mass of the HRP enzyme [1]. These carbohydrates or glycans are composed of N-acetylglucosamine, mannose, xylose, and fucose [9]. Researchers in this area have concluded that incorporation of these glycan groups confers an additional level of stability to the HRP enzyme molecule [2, 10]. Some of these enzyme stability features attributed to the glycan structures have included: 1) increased heat stability, 2) increased resistance to free-radical formation associated with the enzyme’s 

**Table 1. Common chromogenic HRP substrates used over the years**

<table>
<thead>
<tr>
<th>GENERIC NAME</th>
<th>SCIENTIFIC NAME</th>
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<tbody>
<tr>
<td>ABTS</td>
<td>2,2’-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)</td>
</tr>
<tr>
<td>Benzidine</td>
<td>4,4’-diaminobiphenyl</td>
</tr>
<tr>
<td>TMB</td>
<td>3,3’,5,5’-tetramethylbenzidine</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3’-diaminobenzidine</td>
</tr>
<tr>
<td>OPD</td>
<td>o-phenylenediamine</td>
</tr>
<tr>
<td>MBTH</td>
<td>3-methyl-2-benzothiazolinone</td>
</tr>
<tr>
<td>Guaiacol</td>
<td>2-methoxyphenol</td>
</tr>
<tr>
<td>Pyrogallol</td>
<td>1,2,3-trihydroxybenzene</td>
</tr>
<tr>
<td>Phenol</td>
<td>hydroxybenzene</td>
</tr>
<tr>
<td>p-Cresol</td>
<td>4-methylphenol</td>
</tr>
<tr>
<td>o-Dianisidine</td>
<td>3,3’-dimethoxybenzidine</td>
</tr>
<tr>
<td>p-Toluidine</td>
<td>1-amino-4-methylbenzene</td>
</tr>
<tr>
<td>Tolidine</td>
<td>3,3’-dimethylbenzidine</td>
</tr>
<tr>
<td>Hydroquinone</td>
<td>1,4-dihydroxybenzene</td>
</tr>
<tr>
<td>4-Aminoantipyrrene</td>
<td>4-amino-2,3-dimethyl-1-phenyl-3-pyrazolinone</td>
</tr>
<tr>
<td>5-AS</td>
<td>5-aminosalicylic acid</td>
</tr>
<tr>
<td>4-CN</td>
<td>4-chloro-1-naphthol</td>
</tr>
<tr>
<td>AEC</td>
<td>3-amino-9-ethylcarbazole</td>
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redox activities, 3) increased enzyme solubility in aqueous medias, and 4) creation of an increased structural rigidity feature helping to combat heat and other adverse environmental conditions [2]. These hostile environments can lead to the unfolding/denaturation of the tertiary structure of the enzyme. Using X-ray crystallography, a three-dimensional image of HRP-C was obtained [11]. This revealed an α-helical folding structure with a small section of β-sheet. HRP-C consists of two domains with a hydrophobic pocket located between them. Within the hydrophobic pocket lies the heme prosthetic group (ferriprotoporphyrin IX) whose function is to conduct the enzyme oxidation and reduction (redox) activities [2, 6, 10]. A more comprehensive explanation of heme associated redox electronics as it relates to the oxidation of 3,3',5,5'-tetramethylbenzidine (TMB) will be provided in the final section of this technical presentation.

Predecessor chromogenic substrates for HRP

Early recognition of HRP as a stable and reliable oxidizing enzyme and subsequently a useful enzyme redox component for immunodiagnostic purposes, lead to the development early on of many chromogenic substrates. Each new substrate formulation was intended to provide a greater signal sensitivity than its predecessors. In Table 1, we list some of the chromogenic HRP substrates that were routinely used for HRP detection over the years. A review of past chromogenic HRP substrate publications reveals a few multiple dye comparison studies of HRP substrate dyes that were in routine use for diagnostic purposes. In one multiple oxidized dye sensitivity comparison, going from highest to lowest sensitivity, they observed the following sensitivity hierarchy: TMB > OPD > ABTS > 5-AS > MBTH > AEC [12]. An earlier study comparing the detection sensitivities of the OPD, ABTS, o-dianisidine, and 4-aminoantipyrine HRP substrates found a sensitivity hierarchy consisting of OPD > ABTS > o-dianisidine > 4-aminoantipyrine [13]. Combining these published sensitivity comparisons with other published anecdotal accounts, we conclude that for the contemporary HRP chromogenic substrates, the relative detection sensitivity hierarchy would be as follows: TMB > OPD > ABTS.

Additional motivation for the derivation of newer and improved chromogenic HRP substrates arose from a desire to move away from a dependence on benzidine analog composition dyes. Benzidine is classified as a known carcinogen [14]. Its conversion to its carcinogenic form via o-hydroxylation of its aromatic amine structure by liver based oxidases was proposed by Josephy et al [15]. Peroxidase catalyzed oxidation of benzidine can lead to formation of reactive electrophiles which may facilitate its binding to DNA [14, 15]. Using Salmonella enterica serovar typhimurium TA 1538 as the reference indicator organism within an Ames testing format,
several benzidine analogs were analyzed for their mutagenic potential [16]. In the presence of liver microsome sourced enzymes (oxidases) as part of an Ames testing format, benzidine and benzidine derivatives such as o-dianisidine, were found to induce frame-shift mutations in the *Salmonella* typhimurium TA 1538 indicator organism. In contrast, the TMB substrate did not exhibit any mutagenic properties [16]. This was the logical mindset leading to the derivation and synthesis, using 2,6-dimethylaniline as the starting material, of a benzidine derivative having all four of its o-positions blocked by a methyl group (TMB) [17]. TMB was further demonstrated to have little carcinogenic activity in rats [17]. A structural comparison of benzidine and its DAB and TMB derivatives is illustrated in Figure 1. In 1981, Bos et al. published and promoted the use of TMB as a non-mutagenic signal generation substrate for enzyme immunoassay (EIA) formats [18]. Over subsequent years, multiple investigators put forth a substantial effort toward addressing: 1) limited TMB solubility in aqueous solution properties, 2) long term TMB substrate storage stability limitations, and 3) instability of oxidized blue-green and yellow oxidation state substrate solutions [19-21].

To further expand on this TMB substrate optimization discussion, efforts were made to modify TMB substrate formulations to maximize oxidized TMB (colored product) detection sensitivity. A major emphasis was also put toward being able to provide an absorbance-stable acid stopped, yellow-colored TMB-oxidation-product. This process would involve multiple TMB formulation adjustments. Providing a TMB substrate product with an extended time stable A450 absorbance property is an absolute imperative when running staggered multiple ELISA plate assays or when using an automated ELISA system. These TMB performance optimization efforts have also led to our product’s excellent long-term storage stability (> 2 years at 2 - 8°C). ICT offers several TMB 1-Step type product formulations (H2O2 and TMB provided within a common solution) that help you target your optimal ELISA detection sensitivity range. These include the TMB Supersensitive 1-Component HRP substrate formulation (SUBS, Catalog # 6275), the TMB 1-Component HRP substrate formulation (SUBT Catalog # 6276), and the TMB Slow-Kinetic 1-Component HRP substrate formulation (SUBK Catalog # 6277). All ICT brand HRP and Alkaline Phosphatase, ELISA and insoluble precipitate forming membrane substrate products, provide the end-user with a long-term storage stable, sensitive, safe, and user-friendly research tool.
Hydrogen peroxide ($\text{H}_2\text{O}_2$) as oxidant for HRP redox cycle initiation

Initiation of an HRP enzyme-mediated color development process for signal generation is dependent upon the presence of $\text{H}_2\text{O}_2$ as the oxidizing agent to initiate the redox process (Figure 2). $\text{H}_2\text{O}_2$ consists of two OH molecules with the two oxygen molecules covalently bonded via a single O-O bond (Figures 2 and 3) [22]. Intracellular reduction of $\text{H}_2\text{O}_2$ via $\text{Fe}^{2+} \rightarrow \text{Fe}^{3+}$ (Fenton reaction) leads to the production of toxic reactive OH $+$ •OH radicals that are capable of reacting with other key intracellular components such as DNA, proteins or membrane lipids [23]. Its ability to perform as an oxidizing agent is attributed to the relatively low bond energy of the peroxide (peroxo) O-O covalent linkage. The energy requirement for disruption of this peroxide (O-O) bond was calculated to be $\sim 45$ kcal/mol compared to bond disruption energies for C-O and N-O linkages at 84 kcal/mol and 53 kcal/mol respectively [24]. This low O-O bond energy factor enables $\text{H}_2\text{O}_2$ to act as a potent two-electron electrophilic oxidizing agent as evidenced by its high (1.77 V) redox potential [25]. $\text{H}_2\text{O}_2$ migrates into a large cavity on the heme-distal side region that forms a small 3Å by 3Å cylindrical pocket where it binds to and reacts with the heme redox site [26]. A more detailed description of the $\text{H}_2\text{O}_2$ mediated oxidation of the HRP will be presented in the next section.

Part 2

Redox relationship linking $\text{H}_2\text{O}_2$ to HRP to TMB

HRP plays a central catalytic role in many enzyme immunoassay (EIA) signal generation schemes. Possession of a catalytically efficient, redox capable, enzyme-prosthetic group structure is a mandatory prerequisite for its centralized substrate processing role. In this section, we list the key reaction steps that are driving the HRP oxidation and reduction processes. A logical starting point for describing the interactive chemical relationships between HRP and its oxidizing agent ($\text{H}_2\text{O}_2$) and its reducing agent (TMB), would be to split this discussion topic into two subsections. To this end, we broke this HRP redox chemistry discussion into two parts; 1) redox cycle initiation involving $\text{H}_2\text{O}_2$ as the oxidizing agent and 2) the redox cycle completion reaction involving TMB as the electron donor reducing agent.
**HRP enzyme redox cycle driven by H$_2$O$_2$ and TMB**

The first subsection summarizes the key H$_2$O$_2$ initiated heme oxidation reactions. The second subsection summarizes the key chemical interactions between HRP and TMB during the HRP reduction process. A generic enzyme redox reaction pathway defining both the oxidation and reduction steps within our HRP redox cycle, can be represented by four generic reaction sequence equations:

1. Resting state enzyme + H$_2$O$_2$ → Cpd-I + H$_2$O
2. Cpd-I + AH → Cpd-II + AH$^•$
3. Cpd-II + AH → resting state + AH$^•$
4. Overall reaction → 2AH + H$_2$O$_2$ → 2H$_2$O + 2AH$^•$

The AH$^•$ in this HRP redox cycle discussion can be represented by the TMB substrate.

The resting state enzyme above would be represented by the HRP Fe$^{3+}$ or Fe (III) heme form.

A simplified summary figure of this HRP redox cycle is illustrated in Figure 4 below.

We used TMB as the reducing substrate example in this discussion because it is the electron donor/chromogenic component in the H$_2$O$_2$ + HRP + TMB redox reaction cycle. It should be stated however that because of HRPs notoriously low specificity for compatible electron-donor-substrate candidates, it became possible over the years for the development of many chemical-structure-variable chromogenic substrates [27]. That said, TMB is just one of multiple chromogenic HRP-compatible electron donor candidate as seen in Table 1 above.

**HRP oxidation process initiates the redox cycle**

Activation of the HRP redox cycle is made possible via a two-electron oxidation of HRP event that is initiated by its oxidizing substrate H$_2$O$_2$. Occurring as a two electron oxidation of the heme prosthetic group, native resting-oxidation-state [Fe$^{3+}$ or Fe (III)] heme is converted into a high oxidation state [Fe$^{4+}$ or Fe (IV=O)] oxoferryl species that is typically referred to as Cpd-I [1, 6]. This two-electron oxidation event courtesy of the H$_2$O$_2$ oxidizing substrate leads to the formation of the heme intermediate oxidation state Cpd-I. It is the oxidizing power of Cpd-I that serves as the driving force behind the conversion of colorless TMB substrate into the blue-green colored oxidation product used for signal generation in ELISA type EIA formats.

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**Figure 4.** Hydrogen peroxide (H$_2$O$_2$) driven initiation of the horseradish peroxidase (HRP) redox cycle via the creation of high oxidation state Fe(IV=O) Cpd-I. This two electron oxidation equivalents bearing heme-Cpd-I acts as the driving oxidative force behind the conversion of colorless TMB starting material into its blue-green one electron oxidation state product. Along the redox cycle pathway, Cpd-I is subsequently reduced via a molecule of TMB forming a second lower oxidation potential oxidizing heme intermediate in the form of heme-Cpd-II. Heme-Cpd-II is subsequently reduced via another molecule of TMB returning the heme to its Fe (III) resting state form.
HRP driven TMB color development

Oxidation chemical reaction steps of the HRP redox cycle

1. The generalized reaction of H₂O₂ with the native resting oxidation state heme can be defined by a three-reaction sequence.
   a. Heme + H₂O₂ → Heme-H₂O₂ = hydroperoxo-ferric complex
   b. Heme-H₂O₂ → Cpd-0
   c. Cpd-0 → Cpd-I + H₂O along with conference of a 2-electron oxidation equivalence to the Cpd-I intermediate.

2. Initial binding of the neutral-charge-form of H₂O₂ to the resting state heme [Fe³⁺ or Fe (III)] occurs within the distal heme pocket as defined by Arg38, Phe41, and His42. This binding event leads to the formation of the hydroperoxo-ferric complex (heme-H₂O₂) [2, 28].

3. Heme-H₂O₂ converts to Cpd-0 as the result of a His42 facilitated deprotonation of the H₂O₂ while still bound to the Fe (III).

4. Cpd-I is then formed by the heterolytic cleavage of the peroxy (O-O) bond after back protonation via His42 of the distal OH group on Cpd-0. Cleavage of the peroxy bond results in simultaneous reduction of the H₂O₂ to H₂O with concurrent 2 electron oxidation of Cpd-0 to create the Cpd-I intermediate [28, 29]. This reaction requires the joint participation of His42 and Arg38. Arg38’s role in the heterolytic cleavage of H₂O₂ is to lower the pKa of the His42 as well as align the H₂O₂ within the reactive site. The role of His42 is to cleave the H₂O₂ (O-O) bond resulting in a 2-electron oxidation event that converts Cpd-0 to Cpd-I. Two electron equivalents are simultaneously transferred to the H₂O₂ oxidizing agent subsequently reducing it to H₂O. This reaction requires a neutral to slightly basic pH environment to put the His42 in its neutral form and Arg38 in its cationic form [28, 29].

5. Cpd-I exists as a high oxidation state [Fe⁴⁺ or Fe (IV=O)] oxoferryl containing heme intermediate bearing two equivalents of oxidizing potential [1, 2, 6]. The 2 positive charges are distributed between the oxoferryl center and a porphyrin ring-based cation radical. The oxidative capacity of Cpd-I becomes the driving force perpetuating the subsequent HRP dependent TMB oxidation process.

Reduction chemical reaction steps of the HRP redox cycle

Completion of the HRP redox cycle is made possible via a two-electron reduction of HRP at the expense of its TMB substrate. Occurring as two consecutive single electron oxidation reactions, colorless TMB substrate is converted into its blue-green colored single-electron oxidation state product. When H₂O₂ oxidized-HRP contacts the colorless unoxidized TMB starting material, the TMB can act as an electron donor substrate converting oxidized HRP back into its reduced Fe (III) oxidation state native form. In so doing, this two-electron acquisition event (reduction) at the expense of TMB, completes the HRP enzyme’s internal redox cycle. Once HRP is converted back into its native Fe (III) oxidation state, it is free to once again participate in another redox cycle with the aid of its H₂O₂ and TMB respective oxidizing and reducing substrates.

Reduction process converting heme back to native Fe (III) form

1. The HRP Cpd-I [Fe⁴⁺ or Fe (IV=O)] reduction process converting heme back to its native (Fe³⁺) ferric oxidation state form can be defined by two reaction sequences.
   a. Cpd-I + AH₂ → Cpd-II + AH•
   b. Cpd-II + AH₂ → the ferric resting state enzyme + AH• + H₂O

In these two reaction sequences, AH₂ represents a reducing (electron donor) substrate and AH•, the oxidized radical form of the reducing substrate. In this discussion, TMB substitutes in for AH₂, acting as the electron donor substrate [29, 30].

2. Cpd-I is a high oxidation state oxidizing agent existing as a Fe⁴⁺ or Fe (IV=O) oxoferryl species containing heme intermediate. As a result of its two-electron oxidation by H₂O₂, it bears two oxidizing equivalents of oxidizing potential. As stated above, the 2 positive charges are distributed between the oxoferryl center and a porphyrin ring based cation radical [1].

3. Cpd-I can be converted to Cpd-II, which also exists as an oxoferryl species, by a one electron transfer step from an electron donor substrate, which in this case is TMB.

4. Cpd-II can be converted back into its Fe³⁺ or Fe (III) (native ferric state) following a second one electron transfer step from another TMB electron donor substrate. In so doing, another H₂O is liberated in the process [6, 26].
HRP-mediated TMB oxidation and color generation

Over a span of almost four decades, HRP has proven quite useful in its ability to reliably conduct the critical redox activities that are required of any chromogenic substrate-dependent signal generation processes. Its low substrate specificity requirements, high substrate turnover rates, and robust stability properties made it the type of enzyme that would make it conducive to its incorporation into many EIA diagnostic testing formats.

In 1982, Josephy et. al devised and published one of the first reaction schemes defining the TMB oxidation product intermediates that occur as the result of the HRP oxidation process [15]. Using electron spin resonance and optical spectroscopy methods, they identified the presence of a TMB semiquinone-imine cation free radical and the associated Charge Transfer Complex (CTC). They envisaged the molecular reaction scheme shown in Figure 5. Within this TMB oxidation scheme there exists two chemically distinct, one electron TMB oxidation products. These consist of: 1) a TMB radical cation and 2) the CTC which is made up of a diamine form electron donor and a diimine electron acceptor. This diamine/diimine charge transfer complex is in rapid chemical equilibrium with the cation radical. Within this dynamic reaction scheme there exists three different reaction component entities; each with its own separate molar absorbance max properties. These reaction component entities consist of the native TMB starting material (diamine form) having an absorbance max of 285 nm followed by the CTC having an absorbance max of 652 nm and finally the two-electron loss/complete oxidation state diimine form with an absorbance max of 450 nm.

The Josephy group also made the experimental observation that if you use a large enough molar excess of \( \text{H}_2\text{O}_2 \) over the TMB (diamine form) starting material, it was possible to convert all the TMB (diamine form) into the two-electron loss oxidation state diimine form. By combining this fact with the observation that each of the individual TMB, CTC, and diimine oxidation state derivatives possess unique molar absorptivity properties, the Josephy group was able to determine the molar absorptivity constants for both the CTC and diimine oxidation state forms. They assigned a \( \epsilon_{450} = 5.9 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1} \) to the diimine form and a \( \epsilon_{552} = 3.9 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1} \) to the CTC form. These two molar absorptivity determinations have been accepted as fact by all future investigators and commercial distributors of TMB substrate products.

Figure 5. 3,3',5,5'-Tetramethylbenzidine (TMB) oxidation product intermediates formed following horseradish peroxidase (HRP) oxidation of the native diamine form of TMB substrate solution. HRP removal of a single electron from the native diamine TMB form leads to the creation of two new intermediate oxidation-state products. These consist of a colorless TMB Cation radical that is in rapid chemical equilibrium with a second blue-green colored intermediate oxidation state entity identified as the Charge Transfer Complex (CTC). The CTC is composed of two different TMB molecules; a colorless diamine form (electron donor) and a yellow colored diimine form (electron acceptor). Following a second HRP facilitated one electron oxidation event, the green TMB product is converted to the yellow colored diimine oxidation product. The CTC has an absorbance max at 652 nm while the diimine oxidation state form displays a maximum absorbance at 450 nm. These two one-electron loss and two-electron loss oxidation state TMB products account for the pre-acid stopped blue-green and acid stopped yellow colored TMB solutions.
Acid amplification of TMB absorbance signal

It is customary in most routine ELISA protocols using TMB as the chromogenic substrate to stop the TMB color development process by the addition of an iteration of acidic stopping solution. The intent here is to create a low enough pH environment from addition of the acid stop solution to shut down the HRP redox activity. In some scenarios (typically involving larger numbers of ELISA plates being tested) it may not be feasible to read each plate immediately after a fixed predetermined color development time frame. It is therefore necessary to stop the catalytic HRP substrate oxidation process to allow completed ELISA plates to be read as soon as time permits.

It was noted early on that a partial oxidation of TMB lead to the production of a blue-green reaction product and complete oxidation of TMB lead to a yellow reaction product [15, 31]. Bally and Gribnau sought to provide an explanation for the significant A$_{450}$ absorbance increases typically observed upon acid stoppage of HRP-mediated TMB oxidation reactions. In one experiment, using UV/VIS (200 nm – 700 nm) spectral scanning methods for monitoring the oxidation product accumulations, they evaluated an HRP-mediated TMB oxidation reaction that proceeded until the A$_{650}$ absorbance values plateaued. At this point, the reaction pH of 5.5 was lowered to pH 1.0 with 2N H$_2$SO$_4$. Upon superimposing the pH 1.0 absorbance spectrum over the pH 5.5 absorbance spectrum, the oxidation state product composition within each pH environment was clearly revealed (Figure 6). Within the pH 5.5 reaction environment, one could clearly identify both an A$_{450}$ diimine absorbance peak as well as an A$_{650}$ Charge-Transfer-Complex (CTC) absorbance peak. Upon acid conversion of the pH 5.5 reaction environment to a pH 1.0 reaction environment, the A$_{650}$ CTC absorbance peak was eliminated. In contrast, the A$_{450}$ diimine absorbance peak was visibly enhanced by what the authors estimated to be 3.2-fold multiplier.

In a different experimental setting, the authors sought to create an HRP-mediated TMB oxidation environment containing a large excess of HRP as well as equal molar concentrations of H$_2$O$_2$ and TMB. Their goal in this case was to achieve a rapid and complete conversion of TMB into its A$_{450}$ absorbing diimine form. As was the case in the previous reaction scheme, this initial reaction was performed at pH 5.5. Once again UV/VIS (200 nm – 700 nm) spectral scanning methods were utilized to monitor the time point at which the A$_{450}$ diimine absorbance peak was maximized. At this time point the pH 5.5 reaction conditions were reduced to pH 1.0 by the addition of 2N H$_2$SO$_4$ and another spectral scan was performed to visualize in this case a second single A$_{450}$ diimine oxidation product. Upon superimposing the pH 1.0 absorbance spectrum over the pH 5.5 absorbance spectrum, the change in molar extinction coefficients between the two pH conditions was revealed (Figure 7). Since the same A$_{450}$ diimine oxidation product is common to both the pH 5.5 and pH 1.0 reaction environments, any differences in the A$_{450}$ diimine absorbance values is the direct result of the different pH conditions in which the two spectral scans were taken (Figure 7). As the net result, it was determined that the acidified (pH 1.0) conditions were directly responsible for increasing the molar absorptivity constants by a factor of 1.4.

Figure 6. Acid induced shift in the chemical equilibrium governing the formation of TMB oxidation products. Acidification of TMB oxidation product reaction conditions going from slightly acidic (pH 5.5) dotted line to highly acidic (pH 1.0) solid line, lead to the complete dissociation of the blue-green colored Charge Transfer Complex (CTC) with a corresponding increase in the yellow colored diimine oxidation product. By shifting the TMB oxidation product equilibrium away from formation of the green colored CTC oxidation product and toward exclusive formation of the yellow colored diimine product, the A$_{450}$ absorbance signal was observed to increase 3.2-fold over that associated with the pH 5.5 A$_{450}$ absorbance signal. Figure image from Bally, R.W. and T.C. Gribnau. Some aspects of the chromogen 3,3',5,5'-tetramethylbenzidine as hydrogen donor in a horseradish peroxidase assay. J Clin Chem Clin Biochem, 1989. 27(10): p 791-6.
Summary of acid induced TMB absorbance enhancement study

1. Lowering the pH from 5.5 (dotted line) to 1.0 (solid line) leads to complete disassociation of the blue-green CTC oxidation product. This action corresponds with an immediate shift in the reaction equilibrium to instead favor formation of exclusively the completely oxidized yellow diimine product. This event is validated by a 3.2X concurrent increase in the A$_{450}$ absorbance peak associated with formation of additional quantities of the diimine oxidation state form (Figure 6).

2. It was also found that lowering the pH of completely oxidized (yellow colored) diimine oxidation product from 5.5 (dotted line) to 1.0 (solid line) conferred a 1.4X increase in the yellow diimine product’s molar extinction coefficient (Figure 7).

3. The final yellow diimine form A$_{450}$ absorbance increase represents the culmination of the acid induced 1.4X increase in the yellow diimine molar extinction coefficient ($\varepsilon_{450}$) plus the acid environment shift in the TMB oxidation product equilibrium leading to the dissipation of the CTC oxidation product in favor of the formation of a corresponding amount of the yellow diimine product. The summation of these two events was used to justify the 3.2X increase in acid induced A$_{450}$ absorbance as depicted in Figure 6.

Part 3

Generalized summaries of key subject matter

This review summary of the interactive chemical relationships existing between HRP and its H$_2$O$_2$ and TMB substrates, represents our attempt to provide a little deeper insight into the chemical dynamics occurring within routine HRP mediated TMB color development processes. We conclude this technical review effort by summarizing key subject matter points from each discussion topic section.

Desirable performance attributes of HRP

- Excellent enzyme stability properties
- High catalytic substrate turnover rates
- Ease of conjugation to other protein/hapten-like molecules. Contains a low number (4) of chemically accessible and reactive primary amines allowing for controllable conjugation reactions
- Relatively low molecular weight enzyme presenting minimal steric hindrance problems
- High (RZ-value) purity HRP enzyme less expensive on per mg basis than other EIA associated enzymes (e.g., Alkaline Phosphatase).
- HRP can be used for both colorimetric and chemiluminescent signal generation assay formats.

HRP enzyme structural features

- Commercial HRP products consist of the isozyme-C (HRP-C) isotype. This prevalence is likely due to HRP-C being the most abundant isotype form (accounting for roughly 50% of the > 40 identified total peroxidase isozymes).
- HRP is a globular glycoprotein having an $\alpha$-helical folding structure with a small section of $\beta$-sheet. 18%-22% of its molecular weight is contributed by its carbohydrate structure.
- These glycan structures are composed of N-acetylglucosamine, mannose, xylose, and fucose carbohydrates
- HRP-C consists of two domains. Between these two domains is located a hydrophobic pocket in which lies the heme prosthetic group responsible for the enzyme’s redox activities.
- Glycan (carbohydrate) structures contribute to some of the high stability and aqueous solubility properties of HRP. These features include increased heat stability, increased resistance to free radical formation resulting from the enzyme’s redox duties, enhanced aqueous solubility properties, and increased structural integrity.
properties that lend themselves to a more robust enzyme stability in hostile environments.

**Chromogenic substrates for HRP**

HRP happens to fall into the category of enzymes having lower specificity requirements for compatible electron donor substrates. This lack of substrate specificity characteristic lead to the synthesis and use of many different electron donor type dye substrates over the years as illustrated in Table 1. The goal was to create more sensitive substrates with enough colored oxidized product stability to enable their use in various diagnostics formats.

- Early HRP substrates displayed varying degrees of signal detection sensitivity.
- Many of these early substrates were chemical derivatives of the benzidine molecule which made them likely to have mutagenic and carcinogenic properties.
- Tetramethylbenzidine (TMB) was synthesized to be a sensitive and non-carcinogenic replacement for these older HRP substrate formulations.
- Contemporary TMB 1-Component HRP substrate products were created to provide a safe, sensitive, and stable chromogenic signal generation mechanism for use in a range of EIA type assay formats.

**Oxidizing properties of hydrogen peroxide**

- Hydrogen peroxide \( (\text{H}_2\text{O}_2) \) serves as the oxidizing HRP substrate that initiates the HRP redox cycle.
- Existing as a covalently linked two hydroxyl chemical structure where the oxygen molecules are linked by a weak (low bond energy) single O-O peroxide or peroxy covalent bond, the presence of this weak O-O peroxide linkage enables it to act as a potent two-electron oxidizing agent.

**HRP enzyme redox cycle overview**

Our generic enzyme redox reaction pathway defining both the oxidation and reduction steps within our HRP redox cycle can be represented by four generic reaction sequence equations:

- Resting state enzyme + \( \text{H}_2\text{O}_2 \) \( \rightarrow \) Cpd-I + \( \text{H}_2\text{O} \)
- Cpd-I + \( \text{AH}_2 \) \( \rightarrow \) Cpd-II + \( \text{AH}^+ \)
- Cpd-II + \( \text{AH}_2 \) \( \rightarrow \) resting state + \( \text{AH}^+ \)
- Overall reaction can be condensed down to: \( 2\text{AH}_2 + \text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + 2\text{AH}^+ \)

A molecule of TMB may be substituted for the \( \text{AH}_2 \) reactant. Resting state enzyme contains heme in the \( \text{Fe}^{3+} \) oxidation state.

**Oxidation steps leading to Cpd-I formation**

\( \text{H}_2\text{O}_2 \) oxidation of heme prosthetic group can be defined by a three-reaction sequence.

a. \( \text{Heme} + \text{H}_2\text{O}_2 \rightarrow \text{Heme-H}_2\text{O}_2 = \) hydroperoxo-ferric complex
b. \( \text{Heme-H}_2\text{O}_2 \rightarrow \text{Cpd-0} \)
c. \( \text{Cpd-0} \rightarrow \text{Cpd-I} + \text{H}_2\text{O} \) along with conference of a 2-electron oxidation equivalence to the Cpd-I intermediate

**Reduction steps returning heme to its native Fe (III) form**

Conversion of high oxidation state (Fe(IV)=O) Cpd-I back to its native resting state Fe(III) form can be defined by a two reaction sequence.

a. \( \text{Cpd-I} + \text{AH}_2 \rightarrow \text{Cpd-II} + \text{AH}^+ \)
b. \( \text{Cpd-II} + \text{AH}_2 \rightarrow \) the ferric resting state enzyme + \( \text{AH}^+ + \text{H}_2\text{O} \)

**TMB oxidation to colored product**

Unoxidized, colorless, native TMB starting material representing the diamine form of the dye (absorbance max at 285 nm) is oxidized by HRP associated Cpd-I intermediate oxidation state heme form. This oxidation event results in the formation of two new one electron TMB oxidation state forms.

a. TMB radical cation
b. Charge transfer complex (CTC) consisting of a colorless diamine form electron donor and a yellow colored diimine form electron acceptor

The diamine/diimine CTC is in rapid chemical equilibrium with a cation radical. Further oxidation of the one electron loss oxidation state CTC form into the two electron-loss oxidation state leads to the formation of the yellow colored diimine form. Within this dynamic reaction scheme there exists three different reaction components each having a different molar absorbance max.

a. Colorless TMB (diamine form) starting material with molar absorbance max of 285 nm
b. Blue-green colored CTC form with molar absorbance max of 652 nm
c. Yellow colored diimine form with molar absorbance max at 450 nm

Exposing TMB to a large excess of \( \text{H}_2\text{O}_2 \) over TMB leads to the complete conversion of all the TMB (diamine form) into the two-electron-loss oxidation state yellow colored diimine form.
**TMB signal enhancement following acid stop of color reaction**

Acid stoppage protocols within routine TMB color development processes reveal an enhancement of the absorbance signal going from the blue-green colored TMB oxidation product to the yellow colored TMB oxidation product. This occurs as the result of the following acid environment driven chemical processes:

a. Complete oxidation, via acid conversion, of the TMB substrate leads to the complete elimination of the blue green $A_{450}$ CTC peak with a concurrent increase in the yellow $A_{450}$ diimine peak. The $A_{450}$ yellow diimine absorbance signal was observed by one group to increase 3.2X over that present before the complete acid conversion step.

b. Reduction of the pH environment from 5.5 to 1.0 of a completely oxidized (all diimine form) TMB solution lead to a 1.4X increase in the diimine oxidation product molar absorptivity constant.

c. The 3.2 fold increase in the $A_{450}$ signal described above, represents the culmination of the 1.4X increase in the yellow diimine molar extinction coefficient ($ε_{450}$) plus the acid environment induced shift in TMB oxidation product equilibrium away from the CTC oxidation product formation to the formation of the yellow $A_{450}$ diimine oxidation product.

We hope that this technical review was helpful in furthering the understanding of the functional mechanism (and history behind) one of the most widely accepted colorimetric EIA signal generation approaches in use today.

**References**

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Resources

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