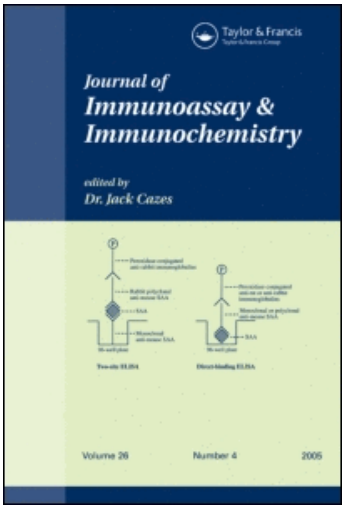


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# A Comparison of Performances of Four Enzymes Used in Elisa with Special Reference to β-Lactamase

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# A COMPARISON OF PERFORMANCES OF FOUR ENZYMES USED IN ELISA WITH SPECIAL REFERENCE TO β-LACTAMASE

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## ABSTRACT

Horse radish peroxidase, alkaline phaosphatase and β-D-galactosidase are widely used as labels in the development of enzyme immunoassays (EIAs). Enzyme β-lactamase, though introduced as a label in late seventies has not yet become very popular inspite of having the necessary features of an enzyme to be used in EIAs. The present article reviews assays developed with this enzyme, highlights its salient features and brings out an argument in favour of its wide spread use in EIAs.

**(KEY WORDS:** Comparison of marker enzymes, Assay parameters, Innovative immunoassays, β-lactamase based assays)



Since the introduction of enzymes as labels in immunoassays by Engvall and Perlman, and Van Weeman and Schuurs , a continuous addition of newer enzymes have been made to the list of enzymes suitable for use in the development of enzyme immunoassays (1,2). The choice of enzyme is largely dependent upon whether a "homogenous" or a "heterogenous" type of immunoassay is to be employed. In the homogenous type of immunoassay, the enzyme in the conjugate behaves differently when it is bound to its specific counterpart hence obviating the need of separation of enzyme labeled reactant before the measurement of enzyme activity. Enzyme malate dehydrogenase (EC 1.1.1.37), glucose-6-phosphate dehydrogenase (EC 1.1.1.49), hexokinase (EC 2.7.1.1), lysozyme (EC 3.2.1.17) and ribonuclease A (EC 3.1.27.5) have been commonly employed in homogenous type of assays, as activities of these enzymes in the conjugate can be modulated when bound to its counterpart (3-10). For example, in a homogenous enzyme

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immunoassay (EIA) for theophyllin, activity of enzyme glucose-6-phosphate dehydrogenase was inhibited up to 90-100% on addition of antibodies to the theophyllin derivative (5).

On the other hand, enzymes in heterogenous type of EIA behaves almost identical in the conjugate (free) and in the immunocomplex (bound), necessitating separation of enzyme labeled reactant from the immunocomplex before the measurement of enzyme activity in either the bound or free fraction. Heterogenous immunoassays are more commonly employed due to the simplicity in the preparation of their labels and relatively higher sensitivity of the resultant assays.

The ten most commonly used enzymes as markers in heterogenous immunoassays are listed in Table 1 and a recent survey revealed that horse radish peroxidase (HRP) is the most popular choice followed by alkaline phosphatase (AP) and β-galactosidase (β-gal) (8-11). In the commercially available diagnostic ELISA kits also HRP is most widely employed (80%), followed by AP (20%) and β-gal. (< 1%) (12). β-lactamase has been extensively used in the authors' laboratory and by several other groups in India. However, application of this enzyme in EIA has not yet become popular. This article reviews the features of the above three enzymes, which might have led to their popularity in diagnostics and highlights the characteristics and performances of β-lactamase based ELISAs. The current article also aims at popularizing its use and stimulating innovative research work such as development of newer and better substrate-chromogen reactions.



## HORSE RADISH PEROXIDASE

### (a) General Features

Horse radish peroxidase (HRP) was the first enzyme used in EIA based on its earlier application in histochemistry (13). The general characteristics of this enzyme are given in Table 2(a). Methods for its conjugation to biological compounds, especially for immunoglobulins have been extensively described (14,15). It has a high turnover number of 66000 $min^{-1}$ and catalyses the reaction of hydrogen peroxide with certain organic, electron donating substrates to yield highly colored products. The reaction of HRP with its substrate, $H_2O_2$, forms a stable intermediate that can dissociate in the presence of a suitable electron donor, oxidizing the donor and potentially creating a color change. A number of substrate--chromogenic reactions are available for its detection (Table 2b).

### (b) Preparation and stability of HRP conjugates

A number of methods have been described for coupling HRP to proteins, each method has its own merits and demerits. The polysaccharide chains of HRP, a glycoprotein,

**Table 1**

**Enzymes commonly used as label in Heterogenous ELISAs**

| ENZYME | EC number | Systemic name |
|---|---|---|
| Horse radish peroxidase | 1.11.1.7 | Donor;Hydrogen-peroxidase – oxidoreductase |
| Alkaline Phosphatase | 3.1.3.1 | Orthophosphoric-monoesterphosphohydrolase |
| β-D-galactosidase | 3.2.1.23 | β-D-galactosidase galactohydrolase |
| Glucose – Oxidase | 1.1.3.4 | β-D-glucose: Oxygen –1-Oxidoreductase |
| Gluco amylase | 3.2.1.3 | Nil |
| Carbonic anhydrase | 4.2.1.1 | Carbonate dehydratase |
| Acetylcholinesterase | 3.1.1.7 | Acetylcholine acetylhydrolase |
| Urease | 3.5.1.5. | Urea amidohydrolase |
| Pyrophosphatase | 3.6.1.1. | - |
| β- lactamase | 3.5.2.6 | Penicillin amido-β-lactam hydrolase |

**Table 2 (a)**

**Essential characteristics of enzymes HRP, AP and β-gal for development of EIA**

| | HRP | AP | β-gal |
|---|---|---|---|
| Monomeric molecular mass | 44,000 | 84,500 | 1,16,250 |
| Protein structure | Monomeric 308 amino acids, 4 disulfide bridges, carbohydrate content : 22% | Dimeric active species, amino groups : 8 – 16 moles/mole enzyme | Tetramer free thiol groups > 12 moles/mole |
| PH optimum | 6 – 7 | 8 – 10 | 5 – 6 |
| PI | 8.7 | 5.7 | 4.6 |
| Specific activity | 4500 $Umg^{-1}$ | 1000 $Umg^{-1}$ at 37°C | 600 $Umg^{-1}$ at 37°C |
| Turnover number | 66000 $min^{-1}$ | 100000 $min^{-1}$ | 3.95 x $10^5$ $min^{-1}$ |
| Specific activity suitable for EIA | > 2000 $Umg^{-1}$ | > 7000 $Umg^{-1}$ | > 800 $Umg^{-1}$ |

**Table 2(b)**

**Substrate-chromogens available for detection of HRP in EIA.**

| Substrate/ Chromogens | Solubility | Color of Product | Wavelength | Ref. |
|---|---|---|---|---|
| $H_2O_2$+2,2'Azinobis (3-ethyl benzthiazoline sulphonate) | Soluble in water | Green | 410/450 nm | 16 |
| *O*-phenyl diamine | Soluble in water | Brown | 492 nm | 17 |
| 3,3,5,5, Tetramethylbenzidine | Soluble in water | Yellow | 450 nm | 16 |
| Diaminobenzidine | Insoluble in water | Brown | - | 18 |

are often used in cross-linking reactions. Proteins can be coupled to HRP by two-step glutaraldehyde method (19). The enzyme, however, gets partially dimerized or even polymerized by treatment with glutaraldehyde and reproducibility in terms of enzyme antibody molar ratios vary (20). The periodate method originally described by Nakane and Kawai has been more popularly used for coupling HRP to immunoglobulins (21). The maleimide method was found to be superior to the periodate method as both peroxidase and antibody activities were well retained in these conjugates (22).

The use of the N-hydroxy succinimide (NHS) ester maleimide cross-linker such as SMCC (succinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate) provided better control over the conjugation process (12). Initially SMCC is reacted with HRP to create a derivative containing sulfhydryl-reactive maleimide group, which is then allowed to react with sulfhydryl containing antibody to obtain antibody-HRP conjugates. Simple and ready-to-use reagents for coupling haptens or proteins or immunoglobulins to HRP are commercially available and efficiency of coupling with any one of these reagents is high and consistent as claimed by Pierce, USA, a leading company in immunological products.



Preservation and stability of enzyme conjugate for its use in diagnostics are considered as two important aspects of EIA standardization. HRP conjugates can be stored with minimal loss of activity if filtered, sterilized, stored at 4°C and handled aseptically. However, freezing of some conjugated monoclonal antibodies has been shown to result in loss of activity (23). This loss of activity may be overcome by addition of 50% (final volume) glycerol to prevent crystal formation. Nielsen has studied stability of freeze dried HRP conjugated antibodies in the presence of a number of additives to preserve its activity (24). Addition of 0.3M trehalose or 0.8% lactoalbumin and 3.2% sucrose resulted in the lowest loss of activity of conjugated antibodies when freeze-dried in glass vials. This study has concluded that the characteristics varied with each antibody conjugate (24). In our laboratory, peroxidase conjugates were sufficiently stable when stored at 20°C in 50% glycerol or 50% ethylene glycol.

(c) Properties facilitating the development of innovative assays

The incorporation of immunoassay reagents into 'dry phase' format is an emerging concept as it facilitates transformation of laboratory based assays into diagnostic test kits. Although the reagent technology may be complex, the tests are simple and convenient to use. HRP is extensively used in 'dry phase' technology because the detection of HRP activity can be compartmentalized. Enzyme glucose oxidase (GO) acts on glucose generating hydrogen peroxide, the substrate for HRP and with chromogen results in generation of color. This property has been exploited by Fuji photo film company which has developed an assay system for detection of hormone thyroxine (25). It is a competitive binding immunoassay

where different layers contain different components of the assay. On applying the sample, its thyroxine and thyroxine-peroxidase from the top layer of paper matrix migrate through the second layer of antibody to thyroxine covalently immobilized on the paper matrix. Competition occurs between the conjugate and sample thyroxine for limited binding sites. The unbound conjugate continues to diffuse through the reflective layer of barium sulfate into the detection zone. The detection layer consists of a gelatin matrix impregnated with orthophenylene diamine (OPD) and GO, a peroxide generating system. The mid-layer contains glucose, which diffuses along with the conjugate into the detection layer where it interacts with GO generating hydrogen peroxide, a substrate for HRP chromogenic reaction. The rate of color formation is read through the clear plastic support and is proportional to the thyroxine concentration in the sample. The 'dry phase' immunoassay based on the 'enzyme channeling immunoassay technique' described by Syva Co. USA has also the combination of HRP and GO (26). Kodak® has developed a photochemical amplification system for HRP mediated immunosorbent assay (27).



HRP was employed in the development of a novel ELISA – "Catalyzed reporter deposition" (CARD) described by Bobrow et al since HRP is known to catalyze the oxidative condensation particularly dimerization of phenolic compounds by a free radical mechanism (28-30). The application of CARD in a 'sandwich' ELISA as a means of amplification was carried out one step prior to the addition of chromogenic substrate. In the assay where HRP was the immobilized enzyme, a labeled substrate such as biotinyltyramide was added for short time, resulting in the covalent deposition of biotin on the solid phase. After washing, the biotin was then probed with appropriate strepavidin enzyme conjugate with another incubation, and later detected with appropriate substrate. Application of CARD to a variety of ELISA formats has enabled improvement in assay sensitivity up to ten-fold.

HRP and GO have the distinction of being the only two enzymes used also in homogenous immunoassays. In this novel system, monoclonal antibodies with specificities against two different epitopes on the antigen and each labeled either with HRP or GO on reaction come in proximal vicinity (31). Hydrogen peroxide is generated by GO in a reaction with glucose present in the medium, which becomes the substrate for HRP. The local concentration of hydrogen peroxide becomes high enough for HRP and antigen gets detected. The labeled-antibodies in absence of the antigen will be widely apart and the activity of the second enzyme (HRP) will be very limited.

**(d) Conclusion**

In conclusion, HRP could be considered as an ideal enzyme for use in ELISA. Its conjugates have been reported to be superior when compared to AP and β-D-gal. However, all its water-soluble substrates are photosensitive and undiluted conjugates are less stable.

## ALKALINE PHOSPHATASE (AP)

### (a) General Features

Alkaline Phosphatases (APs) are a widely distributed family of isozymes and found in a number of species and tissues. Alkaline phosphatase (EC 3.1.3.1) is considered as the second most popularly used enzyme in immunoassay as it satisfies most of the criteria of a good label (8,9). Table 2(a) gives the characteristics of AP. The active site of AP contains two zinc atoms and a single magnesium atom, both of which are essential for its activity. Substrate- chromogen reaction with AP is carried out in a buffered environment containing small concentrations of these divalent cations required for maintaining optimally active site conformation.

AP conjugates are usually applied in situations where because of the presence of high endogenous peroxidase activity, HRP cannot be used. Like HRP, a variety of substrate chromogen reactions have been described for its detection (Table 3). However, p Nitrophenyl phosphate, being water soluble, is widely used in the ELISA.



### (b) Preparation and stability of AP conjugates

AP has been coupled to other proteins by either one- step or two-step glutaraldelyde method (14,35). However, due to its simplicity one-step glutaraldehyde method is widely used. Heterobifunctional cross linkers like SMCC and N-Succinimidyl 3-(2-pyridyldithio) propionate (SPDP) may also be used for coupling AP to antibody molecules. Ready- to-use reagents for coupling AP to haptens and proteins are available commercially.

The enzyme antibody conjugates are usually stored frozen at -20°C. Freezing (especially, freeze-thaw cycles) is damaging to many enzyme / protein in solution resulting in reduction or total loss of activity. This is a significant problem that is especially troublesome because alkaline phosphatases are particularly sensitive to freezing. Glycerol and ethylene glycol have been generally used to stabilize enzymes, including AP as it lowers the freezing point and inhibits the formation of ice crystals. The presence of aldehydes, peroxides and iron ions in ethylene glycol used affects AP activity and hence the need to use contaminant free ethylene glycol as a stabilizer. Ethylene glycol is recommended because it does not support the growth of microorganisms, prevents sub-unit dissociation of protein and also inhibits proteolysis (36). Like HRP, stabilizers for AP conjugates are also available commercially.

### (c) Properties facilitating the development of innovative assay

This enzyme has not been used in 'dry phase' immunoassay. AP, however, was the candidate used in enzyme amplification system (37,38). In this system, the substrate nicotinamide adenine dinucleotide phosphate (NADP) was converted by AP to $NAD^+$. The

**Table 3**

**Substrate – chromogens available for detection of AP in EIA.**

| Substrate-chromogens | Solubility | Color of Product | Wavelength | Ref. |
|---|---|---|---|---|
| Naphthol-AS-BI-phosphate | Soluble in alcohol | Red | 520 nm. | 32 |
| Naphthol-AS-MX-phosphate | Soluble in alcohol | Red | 520 nm. | 33 |
| p-Nitrophenol phosphate | Soluble in alcohol | Yellow | 405 nm. | 34 |

compound was then reduced to NADH by alcohol dehydrogenase in the presence of ethanol included in the reaction medium. In turn, NADH, in the presence of diphorase was converted back into NAD with simultaneous reduction of tetrazolium salt also present in the reaction medium. This resulted in an accumulation of colored soluble formazan dye, proportional to the concentration of $NAD^+$ generated by AP. The newly formed $NAD^+$ is recycled many times, resulting in a 100- fold increase in sensitivity (38). Johanssons and Bates have extensively worked with AP amplification system and have reported an amplification of 105 -fold (39).

**(d) Conclusion**

In conclusion, AP is favored for its high performance and easy to use immunoassay system, its simple reaction kinetics, a variety of assay systems are available for its detection and relatively low toxicity of its common substrates.

## β-D-GALACTOSIDASE

**(a) General features**

β-D-galactosidase (β-gal) is relatively less popular than HRP and AP because of its higher molecular weight (5,40,000) and slower turnover rate. However, its high sensitivity and ready availability makes it one of the more attractive choice for use in EIAs. Its general characteristics are given in Table 2(a). Only two substrates with colorimetric end points are available for β-gal when used in ELISA (Table 4).

**(b) Preparation and stability of β-gal conjugates**

β-gal can be coupled to any protein by one-step glutaraldehyde method. However, when IgG and β-gal are coupled using this method, highly polymerized conjugates are obtained (42). Moreover, activities of both enzyme and IgG were significantly impaired and non-specific binding of polymerized conjugates to the solid phase was greater than with conjugates prepared by the maleimide disulfide method (42). The activities of β-gal and IgG were retained when conjugates were prepared using the maleimide disulfide method (43).

It has been shown that little Fab' is released from Fab'-β-gal conjugate when stored at pH 6-7 at 4°C for at least one year (14,42). β-gal conjugates have definitely higher stability(44).

**(c) Properties facilitating development of innovative assay**

The use of β-gal in qualitative 'dry phase' EIA has not been reported to date. However, this enzyme is used in 'dry phase' immunoassays using fluorogenic substrate as in Am TDA system (45). This enzyme has been employed in the development of Cloned

Table 4

**Substrate-chromogens available for detection of β-gal in EIA.**

| Substrate/ Chromogens | Solubility | Color of Product | Wavelength | Ref. |
|---|---|---|---|---|
| Nitrophenyl-β-D galactosidase | Soluble in water | Yellow | 410 nm | 40 |
| Naphthol AsBIβ-D-galactopyronoside | Soluble in water | Yellow | 410 nm | 41 |

Enzyme Donor Immunoassay (CEDIA), which exploits recombinant DNA technology. Through this technology, a new strain of E. coli synthesizing large inactive fragments of β-gal (ER, Enzyme receptor) and small inactive fragments of the same enzyme (ED, enzyme donor) were produced. The large ER and ED can spontaneously associate to form active β-gal. The ability to reactivate ER is retained by ED hapten conjugate, but binding of ED hapten by anti-hapten antibody prevents re-association. The effect of anti-hapten antibody in preventing re-association is reversed by the presence of free hapten (46).

**(d) Conclusion**

β-gal has been used because of its easy availability. This enzyme is less popular because of its slower turnover rate. However, the development of CEDIA type immunoassays may boost its use in ELISA.

## β-LACTAMASE

The use of enzyme β-lactamase in immunodiagnostics originated in India from the authors' laboratory. This enzyme was selected for developing EIA for human chorionic gonadotropin (hCG) and human placental lactogen (hPL) for two main reasons (47).

1. This was the only enzyme prepared in India at that time and readily available.
2. All the individual components of one of its detection system (Starch-iodine penicillin reaction) were readily available at relatively low cost.

It is because of these two reasons, it became very popular in India as a label for the development of EIAs (47-68). For almost a decade, the enzyme was extensively and perhaps exclusively used in India. Currently the enzyme is placed amongst the 10 ideal enzymes for EIA (69). Some of its salient features and characteristics discussed earlier, but never in connection with the development of immunoassays are :

**1. Salient Features and Characteristics of β-lactamase**

i. The enzyme β-lactamase I consists of a single polypeptide chain of approximately 270 residues having about 21 lysine residues per mole, which makes conjugation to most proteins easy.

ii. Analytical-ultracentrifugation data indicates that the enzyme has a molecular weight of 28000 dalton, S20w of Ca 2.75 and a diffusion coefficient ratio of $8.28 \times 10^{-7}$ $an^2$ $sec.^{-1}$. A frictional ratio of 1.22 has been calculated and optical rotary dispersion (ORD) and circular dichorian (CD) studies reveal the presence of some secondary structures. Isoelectric focussing of a highly purified preparation resolved the enzyme into 3 components of pI 9.2,9.3 and 9.7.

iii. Even in a highly diluted form, the enzyme is stable at room temperature for several hours and the process of conjugation stabilizes the enzyme further.

iv. The enzyme can tolerate pH ranging from 5 to 9 with no loss in its activity and it has a broad pH optimum between pH 6 and 7.

v. The enzyme has a specific activity of 1 x $10^5$ to 1 x $10^6$ $min^{-1}$ (Comparable to peroxidase) (70,71).

## 2. Methods of Conjugation

Proteins having lysine groups can easily be coupled to β-lactamase as it has 21 lysine groups. Proteins including hCG, hPL, luteinizing hormone (LH), follicle stimulating hormone (FSH), lactoferrin,, amoebic antigen, rabbit and goat immunoglobulins have been coupled to β-lactamase by the one-step glutaraldehyde method. One-step glutaraldehyde method is considered antique for conjugation of proteins. However, this method is widely preferred for coupling β-lactamase to other proteins (Table 5). Two-step glutaraldehyde method resulted in precipitation of β-lactamase and conjugates with lower reactivity than those prepared by the one-step glutaraldehyde method were obtained (73). Assays developed with β-lactamase as label and using one step glutaraldehyde method for labeling were found to be equally sensitive when they were compared with respective assays using either HRP or AP or other isotopic labels (54,60,61,63). It has been observed that a molar ratio of 1:1 between protein and β-lactamase yielded conjugates with higher specific activities (61,63,76). Highest specific activities of β-lactamase-immunoglobulin conjugates have been reported with a molar ratio of 6:12 (mg/mg) as compared to conjugates prepared with molar ratio of 12:12, 3:12 and 1:12 (73). In the authors' laboratory a molar ratio of 1:5 (mg/mg) is preferred (63).

Studies were also directed at characterizing the β-lactamase-immunoglobulin and β-lactamase -avidin conjugates (72). Gel exclusion chromatography on Sephacryl S200 of anti-rotavirus β-lactamase conjugate showed that 65% of β-lactamase activity was eluted in fractions containing 200,000 Da protein, corresponding to β-lactamase-immunoglobulin conjugates while only 35% of β-lactamase activity was eluted in the fractions corresponding to the lower molecular weight of native β-lactamase (72). β-lactamase activity ranging from 77% to 81% was obtained in FSH-β-lactamase and LH-β-lactamase conjugates prepared in this laboratory (77). These observations led the authors' to prepare FSH-β-lactamase conjugates with 2- times molar excess of β-lactamase and thus obviating the need for Sephadex G75 chromatography. The unconjugated β-lactamase did not interfere in the assay. In our laboratory, working dilutions of conjugates thus prepared ranged from 1:20,000 to 1:25,000 and stock conjugates were found to be stable up to a period of 3 years (see below).

**Table 5**

**Methods used for labeling proteins and immunoglobulins with β-Lac**

| Sr No | Labelled with βL | Method of conjugation | ELISA for | Comparison with | Ref |
|---|---|---|---|---|---|
| 1. | Goat anti h-IgG/IgM | One-step Glutaraldehyde | U. urealicum | AP-EIA | 60 |
| 2. | Goat anti h-IgG | One-step Glutaraldehyde | M. hominis | HRP-EIA | 65 |
| 3. | F $(ab)_2$ | One-step Glutaraldehyde | Potato Virus X | HRP-EIA | 61 |
| 4. | Rabbit anti h –IgG | Two-step Ficoll | HIV | RIA | 72 |
| 5. | Goat Anti rota virus IgG | One-step Glutaraldehyde | Microbial antigen | RIA | 73 |
| 6. | Goat Anti hCG –IgG | Maleimide Method (SMCC) | hCG | - | 71 |
| 7. | Soluble amoeba antigen | One-step Glutaraldehyde | Amoeba | | 54 |
| 8. | Chorionic gonadotropin | One-step Glutaraldehyde | hCG | RIA | 47 |
| 9. | h Luteinising hormone (LH) | One-step Glutaraldehyde | hLH | RIA | 63 |
| 10. | h Follicle stimulating hormone | One-step Glutaraldehyde | hFSH | RIA | 63 |
| 11. | Human placental lactogen | One-step Glutaraldehyde | HPL | RIA | 52 |
| 12. | Lactoferrin (hL) | One-step Glutaraldehyde | hL | HRP | 74 |
| 13. | Goat anti filarial IgG | One-step Glutaraldehyde | Filarial antigen | - | 68 |
| 14. | Goat antio M. Tuberculosis | One-step Glutaraldehyde | M.tuberculosis | RIA | 66 |
| 15. | Prolactin | One-step Glutaraldehyde | Prolactin | RIA | 75 |

β-lactamase has also been used in the development of immunoassays for haptens including testosterone (T), estrone glucuronide ($E_1G$), cortisol (C), pregnanediol glucuronide (PdG), progesterone($P_4$), oestradiol and 6β-hydroxycortisol (48,51,57,58,68,78,79,92).Most of the studies have originated from India. Initially the carbodiimide reaction was employed for conjugation of T, $E_1G$ and PdG (48,51,57). In the reaction, carboxyl group of E G or PdG was reacted with 1M aqueous solution of 1-ethyl-3-(3dimethyl aminopropyl) carbodiimide hydrochloride. The intermediate compound, O-cylisourea, thus formed was reacted with the amino group of β-lactamase resulting in a conjugation between the hapten and β-lactamase. The recoveries of steroid, enzyme activity and immunoreactivity of steroid in active fractions were shown to be in the range of 0.8–1.2%, 30-50% and 5-6% respectively (77). The working dilutions of these conjugates in the assays ranged from 1:400 to 1:1600. In an ELISA for cortisol, the working dilution of the conjugate was 1:600 when the carbodiimide method was used for preparing the conjugate (93). In the authors' laboratory, it was observed that when the mixed anhydride reaction was employed, the working dilutions of the conjugates increased and ranged between 1:4000 and 1:5000. In the mixed anhydride reaction carboxyl group of PdG was transformed into a mixed anhydride with secondary butylchloroformide in dimethylformamide to which β-lactamase was added in aqueous solution, causing PdG to form a peptide bond with the amino group of the enzyme. Recovery of steroid, enzyme activity and immunoreactivity of conjugates thus prepared were shown to be 1.0-2.2%, 60-80% and 7-10% respectively. The recovery of β-lactamase activity ranged between 91 and 109% when 4 different molar ratios of $P_4$ and β-lactamase were conjugated using the active ester method (78). The active ester method has also been used for coupling oestradiol , progestrone and testosterone derivatives to β-lactamase in EIAs for (68,78,92).



### 3. Measurement of β-lactamase Activity

A variety of colorimetric methods are available for the measurement of β-lactamase (Table 6). Of these, microiodometric method is best suited for estimating β-lactamase in bound fraction. In this method, β-lactamase hydrolyses the C-N bound in β-lactum ring of penicillin producing penicilloic acid in stoichiometric proportion (fig.1). It has high affinity for iodine and decolorizes the starch-iodine solution. The reaction can be arrested by the addition of 5 M hydrochloric acid.

Penicillin V (phenoxy methyl penicilloic acid) is hygroscopic and auto degradation occurs when exposed to moisture. This results in non-specific decolorization of the starch iodine reagent.

Beiniarz et al has described a new chromogenic system for β-lactamase, which yields water-insoluble colored product (80). The assay is based on a kinetic measurement of the

**Table 6**

**Various substrate- chromogenic reactions available for the measurement of β-Lac in EIA.**

| Substrate/Chromogens | Solubility | Change in color of the reagent | Wavelength | Ref |
|---|---|---|---|---|
| Starch-iodine Benzyl Penicillin | Soluble in water | Decolorization of violet color | 620 nm | 63 |
| Starch iodine penicillin G | Soluble in water | Decolorization of violet color | 570 nm | 73 |
| Iodine –polyvinyl alcohol benzyl penicillin | Soluble in water | Decolorization of violet color | 630 nm | 72 |
| Thioacetylcephalosporin tetrazolium | Insoluble in water | Generation of pink color | 530 nm | 71 |

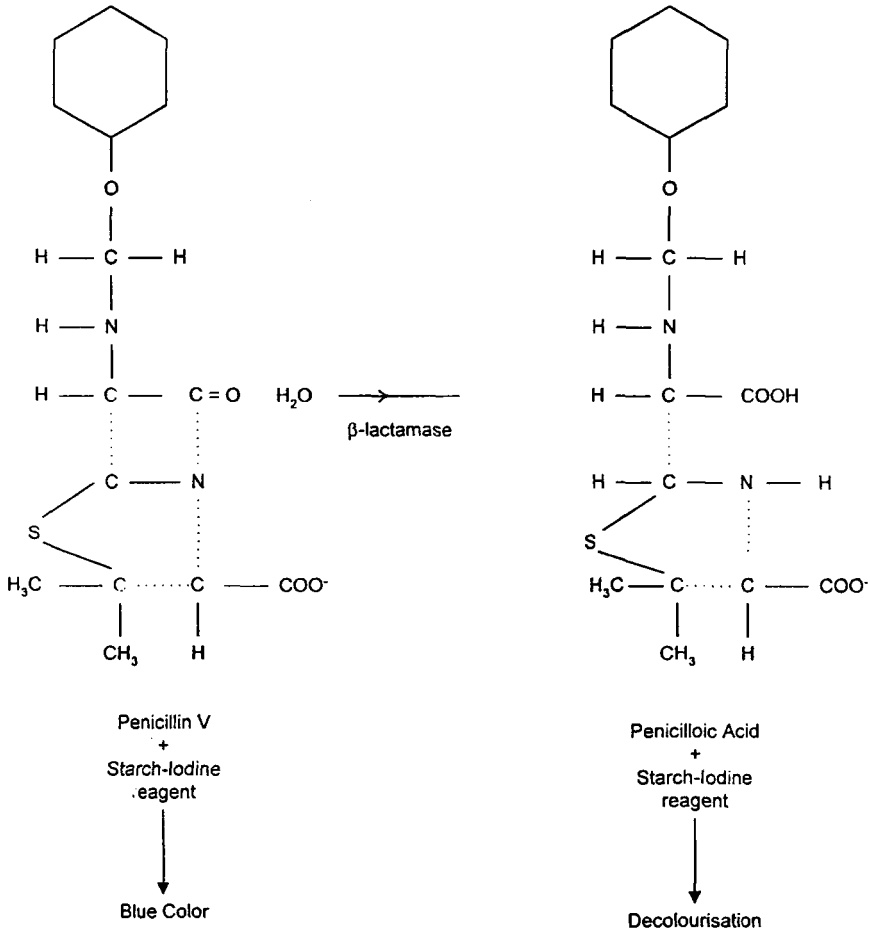


Fig. 1. Colorimetric reaction for the detection of β-lactamase.

appearance of color due to the β-lactamase initiated redox. The substrates are $C_3$' – thiolate – substituted cephalosporine (TAC) which after enzyme- catalyzed hydrolysis of β-lactum-ring undergoes elimination of thiolate ion. The thiolate in the post enzymatic step reduces the tetrazolium salts, which are water soluble colorless compounds to colored water insoluble precipitates of formazan. This new assay for β-lactamase activity is particularly suited for the estimation of β-lactamase on a solid phase such as filter paper, cellulose or any other

immunochromatographic media. Reactions involved are complex and require synthesis of an intermediate compound TAC, which is not available commercially and the stability of this compound is not defined (80).

The starch iodine penicillin (SIP) method is widely used for detecting β-lactamase. This reaction involves decolorization of starch iodine reagent and the reaction has often been criticized. However, Simon et al has highlighted the advantages associated with this reaction (72). The antigen and antibody content of a given sample is determined by measurement of the optical density of a colored end product. In each case a cut-off value has to be defined to discriminate between the induced color resulting from the presence of immunological compound under test and the background of the non-specific reactions. Both types of reactions lead to a colored compound and occur simultaneously. The estimation of acceptable thresholds or cut off values for low titre antibodies or trace levels of antigen is often difficult. Accordingly, the ratio of false positive or negative results strongly correlates with non-specific immunological reactions and thereby with uncertainty of the background for chromogenic reaction developed in currently used ELISA test systems. The starch-iodine reaction of β-lactamase can overcome such methodological problems, since decolorization of color rather than generation of color is involved (72).



**4. Detection limit of β-lactamase conjugates**

The detection limit of an enzyme in the conjugate also governs the sensitivity of the eventual immunoassay. Sauer et al has compared the sensitivity of detection of 4 enzymes viz. HRP, AP, β-gal and β-lactamase in terms of absorbance change per unit time for a given mass of the enzyme. Under the defined conditions of detecting enzyme activities and with approximately 1% co-efficient of variations (precision of assays), β-lactamase was detected with greatest sensitivity (58.8 Δ O.D./ng /h or 1.76 Δ O.D./fmol /h) as compared to HRP (9.92 Δ O.D./ng /h or 0.40 Δ O.D./ng /h), AP (2.48 Δ O.D./ng /h or 0.25 Δ O.D./fmol /h) and β-gal (0.46 Δ O.D./ng /hr or 0.46 Δ O.D./fmol /h) (78). In their method SIP reagent was used for estimating β-lactamase activity. The detection limit of β-lactamase in hCG-β-lactamase conjugate was reported to be $3 \times 10^{-10}$ M (at pH 7.0, 25°c) when an assay based on kinetic measurement of the appearance of color due to β-lactamase – initiated redox reaction was employed (80). In the authors' laboratory, detection limit of β-lactamase in hapten and protein – conjugate ranged between $1 - 1.2 \times 10^{-10}$ M when SIP reagent was used.

**5. Assays developed with β-lactamase**

β-lactamase linked ELISAs were validated using the general criteria described for labeled immunoassays (Table 7).However in this section some of these are discussed in detail.

Table 7

Validation of ELISA developed with β-lactamase

| Analyte | Specificity | | Sensitivity | Accuracy | Precision | | Comparison with RIA | Ref |
|---|---|---|---|---|---|---|---|---|
| | Slope of dose response curve | | | Recovery of added analyte | Coefficient of variation of 3 pools (%) | | Correlation coefficient r = | |
| | Standard | Sample | | (% range) | Intra | Inter | | |
| $E_1G$ | - 2.87 | - 2.70 | 12.5 pg/0.1ml (265 pmol/L) | 85 – 105 | 6.1 – 17 | 3.7 – 11.0 | 0.960 | 51 |
| PdG | - 2.015 | - 2.021 | 12.5 pg/0.1ml (252 pmol/L) | 95 – 102 | < 14 | < 10 | 0.982 | 57 |
| $E_2$ | - 2.076 | - 2.660 | 92 pmol/L | 96 – 108.8 | 3 – 6 | 6 – 13.5 | 0.900 | 68 |
| P | - 1.569 | - | 3.8 pg/tube (121 pmol/L) | - | 5.3 – 11.2 | 6.5 – 10.7 | 0.985 | 90 |
| T | - 1.99 | - 1.90 | 10-15 pg/tube (416 pmol/L) | - | 6 – 8 | 8 – 10 | 0.970 | 48 |
| T | - 1.62 | - 1.16 | 2.5 pg/well (86 pmol/L) | 93 – 105 | 2 – 10 | 2 – 11 | 0.920 | 76 |
| C | - 2.185 | - | 8.1 nmol/L | 89 – 93 | 7 – 14 | 5 – 9 | 0.940 | 58 |
| C | - 2.69 | - | 21.3 nmol/L | 89 – 102 | 5 – 12 | 4 – 9 | 0.920 | 58 |
| FSH | - 2.15 | - 2.07 | 2-3 IU/Ml | 95 – 103 | 6 – 9 | 9 – 12 | 0.904 | 63 |
| LH | - 1.85 | - 2.07 | 2.5-3 IU/Ml | 92 – 104 | 2 – 9 | 7 – 12 | 0.930 | 63 |
| Prl | - 2.660 | - | 2.5μg/L | 101 - 115 | 5 - 13 | 8 – 13 | 0.982 | 75 |

(a) Sensitivity

Amongst the 4 most commonly used enzymes in EIA, β-lactamase has the lowest molecular weight (28000 Da) and highest turnover number ($1x10^5$ to $1 \times 10^6$ $min^{-1}$). Thus fulfilling two of the important criteria to determine the sensitivity of the eventual assay. Conjugates having large enzymes diffuse slowly and may have a greater tendency to bind non-specifically to reaction vessels resulting in "background noise" which in turn may reduce sensitivity of the assay. Assays developed with β-lactamase were equally sensitive when compared directly with radioimmunoassays (Table 8). In HRP labeled EIAs for T sensitivity ranged from 1 to 3 pg/well compared to 2.5 pg/well reported for ELISA using β-lactamase (92).

(b) Precision

The sensitivity of an immunoassay also depends upon the slope of the standard curve and the precision of individual measurement at or near zero-standard. For two assays with the same precision at zero, the assay having steeper curve would be more sensitive. That is, it has the greatest change in measurable response (OD) per small change in dose. The sensitivity and precision though not connected directly by their definitions, are interdependent. Precision of an assay in turn is dependent upon "resolution" or "differences" between the OD reading of two adjacent points on the standard curve. In the authors' laboratory, β-lactamase linked ELISAs have been developed for hCG, hPL, LH, FSH, T, $P_4$, $E_1G$ and PdG wherein the SIP reagent was used for the measurement of enzyme activity in the bound fraction. The differences between OD readings of total binding wells (Bo) and total displacement (NSB) ranged from 1.6 to 2.2. Table 9 shows comparison between OD readings of two assays viz.a protein FSH and a hapten $E_1G$ using β-lactamase and HRP as labels. In case of assays developed with β-lactamase a wider difference is obtained between the OD readings of any two consecutive dose levels, indicating that β-lactamase linked assays have better resolution, which in turn may contribute towards a increased sensitivity. Table 10 shows the OD range of the highest and lowest (Standard-0) and dose levels as obtained with various enzymes and their specific detection systems in few randomly selected ELISAs. It can be seen that in majority of the assays, difference in OD readings remained around 1.0. In β-lactamase linked assays (Table 11), this difference is around 2.0. It is for this reason, β-lactamase linked assays have more potential for its use in visual test assays. In addition its substrate indicator reagent is non-carcinogenic and non-photosensitive. Thus, β-lactamase may be considered as an ideal enzyme for use in qualitative type of immunoassays. β-lactamase based ELISAs had better or equivalent precision when compared with RIA (Table 8).

**Table 8**

**Comparison of validatory criteria of β-lactamase based assays with RIA**

| Analyte | Sensitivity | | Accuracy | | Precision (C.V. of 3 pools) | | | | Ref |
|---|---|---|---|---|---|---|---|---|---|
| | | | % recovery of added analyte | | Inter-assay (range) | | Intra-assay (range) | | |
| | RIA | ELISA | RIA | ELISA | RIA | ELISA | RIA | ELISA | |
| Oestradiol | 37 pmol/L | 92 pmol/L | 86.6 – 93.6 | 96 – 108.8 | - | 3.5 – 5.4 | - | 9.6 – 13.5 | 68 |
| Cortisol | - | 2.13 nmol/L | 82 – 97 | 89 – 102 | 4.6 – 15.2 | 4.5 – 8.6 | 7.7 – 11.8 | 5.8 – 12.2 | 58 |
| Prolactin | 3.5μg/L | 2.5μg/L | 94 – 120 | 101 – 115 | 7.8 – 13.7 | 8.6 – 13.0 | 3.6 – 13.4 | 5.5 – 13.5 | 75 |
| Testosterone | 5 pg/tube | 2.5 pg/well | 93 – 107 | 93 – 105 | 1.7 – 7.3 | 1.7 – 10.3 | 2.0 – 10.4 | 1.4 – 7.3 | 92 |
| Progesterone | 3.8 pg/tube | 11.8 pg/tube | - | - | - | 6.5 – 10.7 | - | 5.3—11.2 | 90 |

**Table 9**

**Comparison of standard curves of FSH and $E_1G$ assays using HRP and β- Lac**

| FSH | | | | | $E_1G$ | | | | |
|---|---|---|---|---|---|---|---|---|---|
| HRP (492 nm) | | | β-Lac (620 nm) | | HRP (492 nm) | | | β-Lac (620 nm) | |
| Dose | Readings | $B/B_o$ | Readings | $B/B_o$ | Dose | Readings | $B/B_o$ | Readings | $B/B_o$ |
| 0 | 1.459 | - | 0.461 | - | 0 | 1.882 | | 0.301 | - |
| 2.5 | 1.419 | 0.93 | 0.608 | 0.92 | 12.5 | 1.729 | 0.89 | 0.533 | 0.91 |
| 5.0 | 1.321 | 0.84 | 0.738 | 0.86 | 25 | 1.608 | 0.81 | 0.843 | 0.79 |
| 10 | 1.207 | 0.74 | 0.950 | 0.74 | 50 | 1.440 | 0.69 | 1.250 | 0.63 |
| 20 | 1.019 | 0.57 | 1.247 | 0.59 | 100 | 1.228 | 0.54 | 1.695 | 0.46 |
| 40 | 0.847 | 0.42 | 1.582 | 0.41 | 200 | 1.004 | 0.39 | 2.030 | 0.33 |
| 80 | 0.637 | 0.23 | 1.881 | 0.26 | 400 | 0.809 | 0.25 | 2.275 | 0.23 |
| 160 | 0.516 | 0.12 | 2.096 | 0.15 | 800 | 0.666 | 0.15 | 2.448 | 0.15 |
| NSB | 0.378 | - | 2.380 | - | NSB | 0.452 | - | 2.881 | - |
| (0-NSB) | 0.981 | - | 1.919 | - | (0-NSB) | 1.430 | | 2.581 | |
| R= | 0.999 | | R=0.991 | | r=0.999 | | | r=0.991 | |
| A= | 3.527 | | A=3.431 | | A=4.39 | | | A =4.42 | |
| B = | -2.47 | | B=-2.35 | | B=-2.19 | | | B=-2.182 | |

A = Intercept B = Slope Dose; FSH=mIU/ml, EIG=pg/0.1ml

**Table 10**

**The optical density range of highest and lowest dose levels in few randomly selected EIAs.**

| Enzymes | Detection System | Type of ELISA | Analyte | Wavelength (nm) | Range of OD | Differences | Ref. |
|---|---|---|---|---|---|---|---|
| HRP | $OPD+H_2O_2$ | NC | Rat Prolactin | 490 | 0.4-1.6 | 1.2 | 81 |
| HRP | $OPD+ H_2O_2$ | C | Progesterone | 450 | 0.2-1.5 | 1.3 | 82 |
| HRP | $TMB+ H_2O_2$ | NC | Hepatitis C virus | 450 | 0.3-0.8 | 0.5 | 83 |
| HRP | $OPD+H_2O_2$ | C | Albumin | 492 | 0.2-0.8 | 0.6 | 84 |
| HRP | $OPD+ H_2O_2$ | AB,C | Thyroxine | 492 | 0.05-0.55 | 0.5 | 85 |
| HRP | $TMB+ H_2O_2$ | AB,NC | Thymulin | 450 | 0.1-0.3 | 0.2 | 85 |
| HRP | $OPD+H_2O_2$ | (a)NC<br>(b)NC,AB | HbsAg<br>HbsAg | 492<br>492 | 0.125-0.7<br>0.125-2.35 | 0.575<br>2.225 | 86<br>86 |
| AP | PNPP | NC | Fibronectin | 405 | 0.1-2.0 | 1.9 | 87 |
| HRP | $TMB+ H_2O_2$ | NC | Interlukin6 | 450 | 0.24-1.2 | 0.96 | 88 |
| HRP | $ABST+ H_2O_2$ | NC | Tumor necrosis factor | 405 | 0.1-1.0 | 0.9 | 89 |
| β-gal | ONBG | C | Progesterone | 405 | 0.01-0.14 | 0.13 | 90 |

NC= Non-competitive ; C= competitive; AB = Avidin-Biotin; ONBG = O-nitrophenyl-β- D -galactopyronoside.

**Table 11**

**Optical density readings range of highest and lowest dose levels in few randomly selected EIAs employing β-lac**

| Detection System | Type of ELISA | Analyte | Wavelength (nm) | Range of OD | Differences | Ref. |
|---|---|---|---|---|---|---|
| SIP | C | Testosterone | 620 | 0.3-2.6 | 2.3 | Authors' Lab. |
| SIP | C | Progesterone | 620 | 0.7-2.5 | 1.84 | C.Das et al (90) |
| SIP | C | Estrone glucuronide | 620 | 0.3-2.8 | 2.5 | Authors, Lab. |
| SIP | C | FSH | 620 | 0.4-2.3 | 1.9 | Authors, Lab. |
| SIP | C | LH | | 0.3-2.3 | 2.0 | Authors, Lab. |
| SIP | C | HPL | 620 | 0.5-2.4 | 1.9 | Authors, Lab. |

**(c) Accuracy**

Accuracy of an assay is judged by the ability of the assay to accurately measure the amount of the analyte added externally to a standard sample. Inter- and intra- assay coefficient of variation of estimates of quality control pools by β-lactamase linked ELISA is comparable to RIA results (Table 8).

**(d) Stability of β-lactamase Conjugates**

In the authors' laboratory, immunodiagnostic ELISA kits for reproductive hormones were prepared using β-lactamase. For haptens ($E_1G$ and PdG), enzyme labels were prepared using either mixed anhydride or carbodiimide reactions and for protein hormones (LH, FSH and hCG ) using a one-step glutaraldehyde reaction. For convenience, these were provided in liquid forms as concentrated solutions. However, they can also be provided in dry lyophilized powder with longer stability.

**1. Checking the Stability of Enzyme Labels**

Throughout the stability study, SIP system was used for detecting β-lactamase activity. In β-lactamase linked ELISAs the time taken for decolorization of SIP reagent is considered as the measure of enzyme activity. Thus, the deterioration in enzymatic and immunological activity of the diluted conjugate will be reflected by an increase in the decolorization time of the total binding wells (Bo), while that of enzyme activity alone, by the time required to decolorize a defined volume of SIP (generally 1 ml) by a fixed volume (50 μl) of the conjugate. Thus, for checking immunogenicity and enzymatic activity of the conjugate, total binding wells (Bo) were set up using preserved and freshly diluted conjugate (control) and the time taken for SIP to decolorize Bo well was compared (91). There was no deterioration of enzyme activity in the conjugate as the decolorization time of the 1ml of SIP reagent with 50 μl of preserved conjugate remained unchanged at each interval of time. In the authors' laboratory, stability of $E_1G$, PdG, LH and FSH conjugates was thus checked. Since decolorization time of Bo wells of these assays did not change substantially over a period of 20 weeks under the defined conditions, it indicated that the immunogenicity of the conjugate remained intact. The study also reported that β-lactamase linked conjugates are stable at 37°C upto 2 weeks (91).

Beiniarz et al has compared the thermal stabilities of β-lactamase alone and when conjugated to goat anti-β-hCG-IgG at 25°C and 45°C (71,80). The results were expressed as percentage of residual enzyme activity at an interval of 5 days ranging from 0 to 30 days. Excellent stability of the conjugate was observed at 25°C, even after 30 days the conjugate displayed 90% of its original activity. In contrast, the enzyme activity of native β-lactamase, under the same conditions was only 60%. Thus, at this temperature, the stability of the

conjugated enzyme was found to be markedly better than the stability of the enzyme alone. In the same study, the conjugated enzyme remained stable at 45°C upto 1 week (71,80).

## KITS DEVELOPED WITH βLACTAMASE AS LABEL

The authors' laboratory in collaboration with the Department of Biotechnology, Ministry of Science and Technology, Government of India undertook the task of developing cost-effective immunodiagnostic kits for reproductive hormones. Enzyme β-lactamase was particularly selected for the following reasons

1. It was found that the linking of β-lactamase to estrone glucuronide, pregnanediol glucuronide, testosterone-carboxymethyloxine,11 alpha hydroxyprogesterone hemisuccinate to enzyme through the mixed anhydride reaction was easy, reproducible and yielded conjugates with high working titres. Protein hormones could easily be coupled to β-lactamase by the one-step glutaraldehyde method yielding conjugates of desired activity.
2. While developing EIAs, it was found that both the immunogenicity, as well as enzyme activity of β-lactamase conjugates did not deteriorate significantly for several years at 4°- 8°C especially in case of hapten conjugates. The conjugates can easily be lyophilized and following reconstitution the enzyme activity is fully recovered, indicating that the process of lyophilization does not inactivate / degrade enzyme. The enzyme β-lactamase is least susceptible to bacterial growth when preserved with appropriate concentration of sodium azide (91).
3. Concentrated solution of starch iodine reagent remains stable up to 9 months. The substrate penicillin V (Phenoxymethyl penicilloic acid) is susceptible to auto degradation in moist atmosphere and needs careful handling. However, in airtight containers penicillin V is stable for 2 – 8 years(91).
4. The price of β-lactamase as per Sigma Chemicals, 1997 is almost similar to AP (Type VII S) and HRP (Type VI). However, the cost for the detection of enzyme β-lactamase using SIP is the lowest. Table 12 depicts the cost of specific components (either chromogen or substrate) of enzyme detection system. Penicillin V, starch and crystalline iodine are prepared locally and other substrate/ chromogens listed in Table 10 need to be imported in India. It fulfills the primary aim of keeping cost of reagents employed (β-lactamase and SIP) low.

## CONCLUSION

Amongst the 3 commonly used enzymes, β-lactamase has the lowest molecular weight, high turnover number and has specific activity comparable to peroxidase. It has about

**Table 12**

**Cost of principal components used in detection system of 4 enzymes**

| Enzyme | HRP | | | AP | | | β-gal | β-Lac |
|---|---|---|---|---|---|---|---|---|
| Component | ABTS | OPD | TMB | PNPP | NAMP | NABP | ONPG | PEN-V |
| Standard Concentration in MM Required | 1 | 4 | 4 | 2.5 | 0.5 | 5 | 3 | 0.4 |
| Cost for 20 ml of reagent (US $, 1997) | 0.438 | 0.015 | 3.231 | 1.285 | 0.371 | 49 | 6.725 | 0.006 |

Based on minimum quantity available from Sigma Chemicals, St. Louis, 1997.

21 lysine residues per mole, which makes its conjugation to most proteins easy. Its activity can be measured using a simple, relatively inexpensive detection system comprising of starch iodine and pencillin V. The enzyme is highly stable at room temperature and the process of conjugation stabilizes the enzyme further. Thus, fulfilling most of the criteria described for an enzyme suitable for use in immunodiagnostics.

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