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Development of Biotin and Horseradish Peroxidase Labelling against Monoclonal Antibody Amyloid-β_{42} and Its Application on Enzyme-Linked Immunosorbent Assay

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Abstract: Alzheimer disease is one of the causes of dementia that could make the patient lose their memories until they unable to do normal activities. It is caused by accumulation of protein in the brain called Amyloid-β_{42} ($A\beta_{42}$), that often used as biomarker for alzheimer by enzyme-linked immunosorbent assay (ELISA). To increase the sensitivity and accuracy, capture antibodies are conjugated with labels such as Horseradish Peroxidase (HRP) and Biotin. However, the two conjugates also differ in sensitivity and specificity, therefore this research is to find which is more optimum and stable for ELISA usage. The molecular mass of the conjugated antibodies was characterized with SDS-PAGE, result shows the pre-conjugated antibody has 150 kDa bands, conjugated antibody with labels is heavier than the pre-conjugated due to the addition of each label. Conjugated antibody tested with tetramethylbenzidine (TMB), then analyzed by ELISA reader. The measurement precision decided with %CV value with results shows dillution from 10^{-1} to 10^{-4} has %CV less than 10%, while Biotin is from 10^{-1} to 10^{-5}. Optimization done by determining fixed antigen with the conjugated antibody, the optimal concentration for HRP is 10^{-3} while biotin is at 10^{-4}. Stability of the conjugated antibody was also determined, HRP measurement started to unstable around second month and third month, while biotin start to unstable around fourth month, this shows the biotin is more stable than the HRP.

Keywords: Alzheimer; $A\beta_{42}$; Conjugated Antibodies; HRP; Biotin; ELISA.

Introduction

Alzheimer's disease is one of the causes of dementia that can result in memory loss and cognitive decline in a person to the point of losing the ability to live independently. Alzheimer's predominantly occurs in the elderly and is a social problem, because families living with members affected by Alzheimer's must deal with uncertainty, fear, and loss of control from exhausting patients (Bruggink et al., 2013). To date, there is no effective treatment for Alzheimer's disease, but there are several therapies and alternative treatments that can slow down the progression of Alzheimer's disease, so early detection of Alzheimer's disease is very important to prevent its onset (Zhou et al., 2016).

Alzheimer's disease is caused by the accumulation of protein plaques in the brain. Amyloid-β (Aβ) is a biomarker often used to detect Alzheimer's disease, which is formed from the cleavage of Amyloid Precursor Protein (APP) by β-secretase and γ-secretase (Selkoe, 1994). Aβ40 and Aβ42 are the most commonly found amyloid components and are neurotoxic to brain nerve cells. These two proteins can be found in cerebrospinal

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fluid (CSF) (Wilczyńska et al., 2020), but CSF extraction is painful for patients.

Previously, Leong and others in 2020, found that Aβ42 is more hydrophobic than Aβ40, which means that Aβ42 is more dangerous because it can aggregate faster than Aβ40 (Leong et al., 2020). This property is used for the diagnosis of Alzheimer's disease, making Aβ42 a common biomarker. In serum, Aβ42 can be detected in small quantities (Bruggink et al., 2013). In addition to Aβ42, there are smaller monomers and fibrils in serum and plasma, so sensors with high specificity and sensitivity are needed to distinguish monomers, oligomers of Aβ42, and these fibrils to detect Alzheimer's disease early (You et al., 2020).

Many techniques are used to detect Aβ42 oligomers, such as enzyme-linked immunosorbent assay (ELISA) (Indra et al., 2017) and Western Blot (WB) (Ida et al., 1996). In general, the ELISA used to detect Aβ is Sandwich ELISA. Sandwich ELISA uses two identical monoclonal antibodies as the Capture Antibody and the Detect Antibody or Reporter Antibody. Several studies have shown that the conjugation of the Detect Antibody in Sandwich ELISA increases the specificity and sensitivity of Aβ oligomer readings (Billingsley et al., 2017; Lakshmipriya et al., 2016; Sakamoto et al., 2018). Commonly used conjugates are Horseradish Peroxidase (HRP) and Biotin. HRP is a label that uses hydrogen peroxide to form a colored solution. Biotin is a label that can bind specifically to Avidin. The Avidin-HRP complex helps increase the specificity of ELISA readings (Hirsch et al., 2002).

In Indonesia, there has not been much exploration of monoclonal antibody conjugation to Aβ42. Each conjugate can increase the signal read by ELISA, especially in samples with very low antigen levels, such as serum. However, the two conjugates also differ in sensitivity and specificity, so further research is needed to investigate the differences between them.

This study is part of the development of monoclonal antibody conjugates against Aβ42. By using HRP and Biotin labels on secondary antibodies in Direct Sandwich ELISA, it is expected to increase the sensitivity and specificity for Aβ42 detection and provide information on the advantages of both labels. Sensitivity and specificity will be validated using ELISA method.

Method

Chemicals

The SureLINK™ (86-00-01) biotin labelling kit was purchased from KPL Scientific, Inc. (Montreal, Canada), while the HRP labelling kit was purchased from Dojindo Laboratories Co., Ltd. (Kumamoto, Japan). The in-house $A\beta_{42}$ monoclonal antibody was obtained from Primate Research Center stock. The amyloid-β_{42} standard solution were purchased from Abcam (Oregon, US).

Conjugation with Horseradish Peroxidase

Unconjugated inhouse antibody prepared in tube filter until 100 µG, then washed with 100 µL washing buffer from the HRP labelling kit, and then centrifuged with 8000 x g for 10 min. The filter then washed again with 100 µL washing buffer and centrifuged with the same settings. 10 µL Reaction buffer then added to NH2-Reactive Peroxidase and immediately added into the washed tube filter with incubation at 37 ℃ for 2 h. The antibody then washed again with 100 µL washing buffer and centrifuged with the same settings. The antibody then dilluted with storage buffer and stored at 0 ℃.

Conjugation with Biotin

20 mg/mL biotin prepared by dilluting 1 mg biotin with 50 µL DMF. The antibody buffer exchanged to modification buffer (100mM phosphate, 150 mM NaCl, pH 7,2-7,4) by dialysis. The biotin solution added to the antibody with the modification buffer by 50 µL, then incubated for 2 h. The antibody then centrifuged to seperate it from the modificatioin buffer with 12000 x g for 30 minutes 4 times, the antibody dilluted with PBS and stored at 0 ℃.

Characterization of Conjugated Antibody with SDS Page

The conjugated antibody and preconjugated antibody are pipette into a 6% acrylamide gel, and then the SDS-PAGE was run in 90 V and 400 mA settings for 90 minutes. The gel was stained with coomassie blue liquid and then was destained.

Colorimetric Characterization of Conjugated Antibody Activity

Conjugate was prepared by making a new stock of 50 µg/µL, and diluted in steps of 7 times. The conjugate was added to the ELISA plate, incubated at 37 ℃ for 1 h, and the plate was washed with 0.05% PBST 4 times. For biotin conjugate, streptavidin was added and then incubated at 37℃ for 1 hour, and the plate was washed 4 times. Both conjugate plates were added with substrate in the ratio of 1:1 (Peroxidase:TMB) as much as 100 µL, then incubated again for 15 minutes at room temperature. The reaction was stopped with 90 µL of 2N H_2SO_4. The color change was observed, and the OD was measured with an ELISA Reader.

Conjugated Antibody Optimization for ELISA

The 50 µg/µL conjugate stock was diluted in increments of 7 times. ELISA plate was added with inhouse antibody without conjugate at a concentration of 20 µg/mL for 100 µL, and incubated overnight at cold temperature. The plate was then washed with 0.05%

PBST 4 times, blocked with 5% skim milk blocking buffer in 0.1% PBST for 300 µL, and incubated at 37°C for 1 hour. Plate was washed with 0.05% PBST 4 times, and added 10 ng/mL Ab42 antigen for 100 µL and incubated at 37 °C for 1 h. The plate was washed with 0.05% PBST 4 times, and treated with biotin-labeled antibody conjugate and HRP, and incubated at 37 °C for 1 h.

The plate with biotin conjugate was added with streptavidin-HRP 1:10000 in PBS for 100 µL, and incubated at 37 °C for 30 min. Both biotin and HRP conjugate plates were washed with 0.05% PBST 5 times, and 100 µL of 1:1 substrate (Peroxidase:TMB) was added, and incubated at 37 °C for 15 min. Then the plate was added with 90 µL of 2N H_2SO_4. The OD of both plates was read with an ELISA Reader

Conjugate Stability

Stock 50 µG/µL dilluted 7 times, then stored at 0 °C. Optical density then measured monthly once with ELISA Reader.

This research follows flow chart from Figure 1.

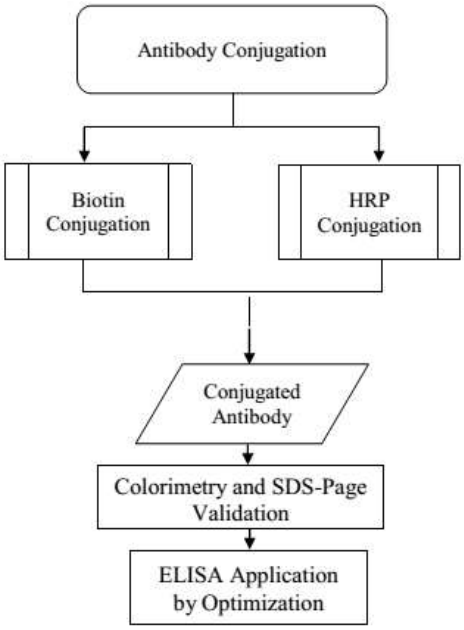

Figure 1. Flowchart on the development of biotin and HRP conjugates against amyloid-β_{42} monoclonal antibodies and their application in immunoassays

Result and Discussion

Conjugation and Characterization of Conjugates with SDS-Page

Horseradish peroxidase (HRP) is an enzyme that is often used as a label in various immunological techniques (Khramtsov et al., 2025). HRP can be conjugated with primary or secondary antibodies. Complexes of HRP with antibodies or other labels can be utilized to detect antigens by enzymatic reactions (Martell et al., 2016). Biotin is a water-soluble chemical compound, which is one of the essential coenzymes for humans (Erbach et al., 2022). Biotin has been used as a label and binds strongly to avidin to form a non-covalent ligand that is fast and undisturbed by pH, extreme temperatures and organic solvents, so biotin is very well used in immunoassays (Balzer et al., 2023). HRP has a molecular weight of about 40 kDa (Greifenstein et al., 2024), while biotin has a molecular weight of 244.31 daltons (Zou et al., 2023).

The reaction between the peroxide label that binds to the secondary antibody and the substrate can increase the sensitivity of immunoassay readings, such as ELISA. There are several compounds that can be used in biochemistry and molecular chemistry to conjugate a label with a protein that has a primary amine group, namely N-hydroxysuccinimidine (NHS), sulfonyl chloride, isocyanate, and isothiocyanate (Huang et al., 2021).

The conjugation of biotin and HRP is done using the reaction of the NHS ester leaving group bound to the label. This NHS ester will react with amines resulting in a nucleophilic reaction, which results in the breaking of the label bond with the by-product N-hydroxysulfosuccinimide (Strachan et al., 2004). This reaction will produce a stable, relatively young bond, and is very specific to the amino group on an antibody or a protein. With these properties, it can reduce interference with other chemical groups contained in the target molecule (Chen et al., 2012). Organic solvents such as DMSO or DMF are also used to dissolve the label because NHS Ester compounds can dissolve in organic solvents (Strachan et al., 2004).

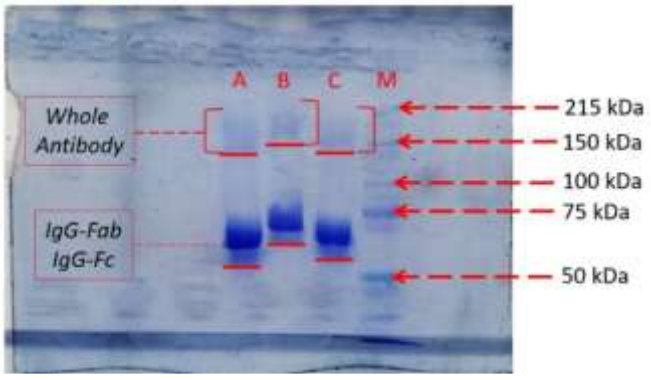

Figure 2. SDS-PAGE Characterization of conjugated antibody and pre-conjugated antibody. M indicate as marker or protein ladder for benchmarking. (A) indicate the pre-conjugated antibody, (B) indicate HRP conjugated antibody, and (C) indicate biotin conjugated antibody

SDS-Page is often used to detect a fraction and covalent aggregates from a sample. Both conjugates and pre-conjugated antibodies were characterized by SDS-

Page, and differences in the weight of the HRP conjugate (fig 2b), biotin (fig 2c), and pre-conjugated antibody (fig 2a) are visible. Typically, monoclonal IgG is present at 148 kDa, as it consists of two light and two heavy chains or called FaB and FaC (Niu et al., 2024). The molecular weight of FaB and FaC are around 50 kDa and 25 kDa, respectively (Hörner et al., 2021; Xu et al., 2024). Each FaC binds to FaB by a disulfide bond, and FaB also binds to other FaBs by two or four disulfide bonds. In this study, Figure 1 shows that there is a band at 150 kDa which is the whole antibody, while the area around 75-50 kDa shows a mixture of two bands which are a mixture of FaB and FaC. It can be seen that the result of conjugation has a greater weight than the antibody before conjugation, where the antibody conjugated with HRP is heavier than the antibody conjugated with biotin. This states that the conjugation is successful, and increases the molecular weight of the conjugated antibody.

Conjugate and Substrate Activity by Colorimetry

Avidin is a protein that has four sides to bind to biotin (Jain et al., 2017). The characteristics of these four sides are different, but also have in common that the β-strands form into a lop that has a place to bind to biotin at the end (McConnell, 2021). The valve on the avidin lop will close, strengthening the bond and lowering the affinity so that it can be used as a good label (Delgadillo et al., 2019). Avidin has several derivatives, one of which is streptavidin. Streptavidin is an avidin derivative that has low similarity primary structures (Balzer et al., 2023). In this study, streptavidin was used to bind biotin and HRP.

Table 1. Colorimetric OD Measurement of HRP Conjugate and Calculation of CV Percentage

Dilution	Absorbance (λ=450 nm)			Mean	SD	%CV
	1	2	3			
Blank	0.013	0.011	0.015	0.013	0.002	12.56
10^{-1}	2.149	2.134	2.121	2.135	0.014	0.54
10^{-2}	2.142	2.120	2.140	2.134	0.012	0.47
10^{-3}	2.106	2.112	2.108	2.109	0.003	0.12
10^{-4}	0.114	0.131	0.125	0.123	0.009	5.71
10^{-5}	0.013	0.010	0.011	0.011	0.002	11.00
10^{-6}	0.003	0.005	0.003	0.004	0.001	25.71
10^{-7}	0.001	0.002	0.002	0.002	0.001	28.28

Table 2. Colorimetric Measurement of OD of Biotin Conjugate and Calculation of CV Percentage

Dilution	Absorbance (λ=450 nm)			Mean	SD	%CV
	1	2	3			
Blank	0.037	0.030	0.032	0.033	0.004	9.00
10^{-1}	2.136	2.161	2.110	2.136	0.026	0.97
10^{-2}	2.158	2.120	2.130	2.136	0.020	0.75
10^{-3}	2.144	2.154	2.105	2.134	0.026	0.99
10^{-4}	2.059	2.065	2.040	2.055	0.013	0.52
10^{-5}	0.482	0.520	0.494	0.499	0.019	3.18
10^{-6}	0.064	0.040	0.057	0.054	0.012	18.78
10^{-7}	0.053	0.030	0.042	0.042	0.012	22.54

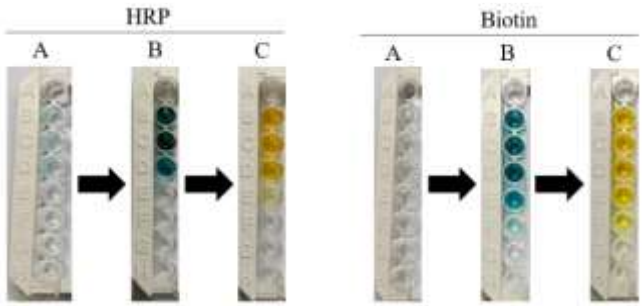

Figure 3. Colour changes that occur (A) before the conjugated antibody is reacted, (B) after reacting with the substrate, and (C) after H_2SO_4 is applied

HRP attached to the secondary antibody will react with the substrate. HRP will catalyze the conversion of chromogenic substrates such as tetramethylbenzidine (TMB) into colored products. TMB is colorless and will be oxidized by H_2O_2, becoming a blue color that can be read Optical Density (OD) with 2 wavelengths, namely 370 nm and 652 nm (Gosling, 2005). After the reaction, the substrate is added acid to produce a stable yellow color change with a wavelength of 450 nm (Mufidah et al., 2015).

In this study, ELISA plates were added with conjugates that had been made, for biotin conjugates added with Streptavidin-HRP 1:10000, and both plates were given TMB substrate and incubated at room

temperature for 5 minutes. If the secondary antibody is successfully conjugated, the plate will produce a blue color. The results showed a change in color to blue and then H_2SO_4 was given so that the color changed to a stable yellow. OD reading of the conjugate that has successfully changed the color of the substrate is done with an ELISA reader at a wavelength of 450 nm. Each conjugate was diluted 7 times, from a stock concentration of 50 µg/µL. The coefficient of variability (CV) value, also called the coefficient of variability, was determined to measure the precision of the measurement. If the CV value is <5%, then the data is acceptable (Desriani et al., 2024). There are two types of coefficient variability that can be determined, namely intra-assay CV and inter-assay CV. The intra-assay coefficient is the result of measurements without variable changes, while the inter-assay coefficient is the result of measurements with variables that can be changed (Reed et al, 2002). The percentage of the intra-assay coefficient if it is <10% (Wong et al., 2004), then the calculation is considered qualified or consistent, while for the percentage of the inter-assay coefficient if it is <15% (Thomson et al, 2014) to be considered qualified. At 10^{-1} to 10^{-4} dilutions of HRP conjugate and 10-1 to 10-5 dilutions of biotin conjugate had %CV <5%, meaning the readings were acceptable at those concentrations.

Conjugate Concentration Optimization for ELISA

Amyloid-β_{42} is a biomarker that is often used in the diagnosis of Alzheimer's using the ELISA method (Rabiei et al., 2025). This protein is soluble in cerebrospinal fluid (Wilczyńska et al., 2020), but is also found in serum (Thomsson et al., 2014), but the signal given is still not high. Conjugates were used as secondary antibodies in this study, in order to increase the signal.

Sandwich direct ELISA is a variation of the ELISA technique that uses two antibodies, capture antibody and detection antibody with an enzyme to produce a measurable signal that easy to operate, and low-test cost (Niu et al., 2024). The complex of the sample and antibody will react with the substrate, which will change color and color intensity, indicating the concentration of antibodies present in the sample (Agrawal et al., 2022; Qin et al., 2020). An in-house antibody with a concentration of 20 µg/mL was used as the primary antibody, and conjugate with a concentration of 50 µg/µL diluted in stages as the secondary antibody, to determine the optimal conjugate concentration.

Each plate is added with $A\beta_{42}$ antigen, and then added with conjugate. The conjugated secondary antibody will attach to the antigen captured by the primary antibody. The concentration of the secondary antibody is varied by diluting it 7 times. The HRP-labeled conjugate curve has an R^2 value of 0.9166 because saturation occurs at low concentrations, namely at a dilution of 10^{-7}. The optimum concentration that can be used is at a dilution of 10^{-3}. The biotin-labeled conjugate curve has an R^2 value of 0.9722 which means that the curve has a good range or more than 0.95 (Lai et al., 2020). The optimum concentration that can be used is at a dilution of 10^{-4} because it has a better %CV than the previous calculation so that the measurement is more stable.

Table 3. Conjugate Concentration Optimization for ELISA Applications

Dilution	Absorbance (λ=450 nm)			
	HRP		Biotin	
	1	2	1	2
10^{-1}	1.801	1.568	2.015	2.102
10^{-2}	1.665	1.367	1.621	1.515
10^{-3}	1.082	1.062	1.523	1.475
10^{-4}	0.480	0.324	1.125	1.185
10^{-5}	0.121	0.101	1.042	0.815
10^{-6}	0.041	0.031	0.452	0.599
10^{-7}	0.019	0.021	0.248	0.225

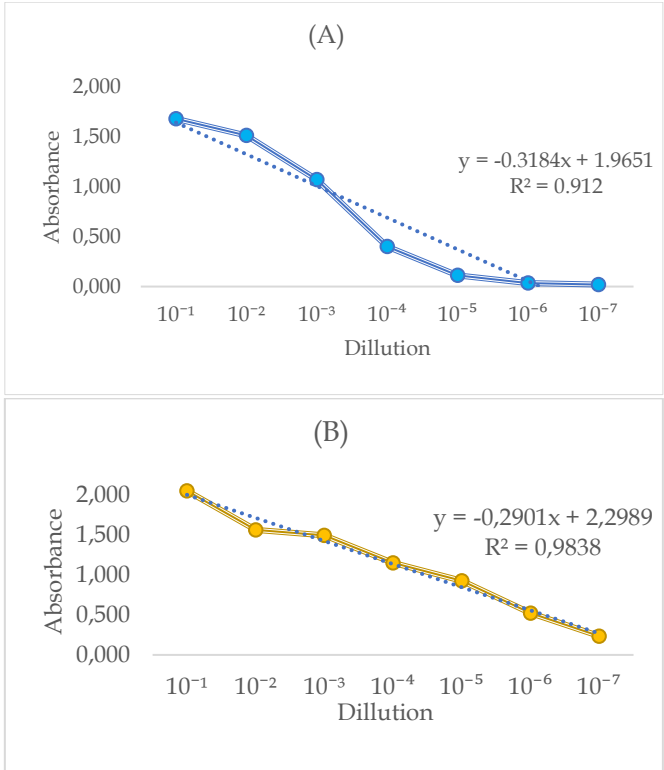

Figure 4. Graph for (A) HRP and (B) Biotin conjugate concentration against absorbance value of ELISA reading

Previously, in 2025, Tirta had conducted readings using the same ELISA system with commercial detection antibodies. The results of the readings at the same wavelength of 450 nm, with in-house antibodies and the same concentration of 20 µg/mL, produced OD values of 0.1560 and 0.2250. This proves that conjugation with

antibodies similar to the capture antibody used increases the sensitivity of the ELISA. Thus, the use of antibody reagents can be reduced and can be used with optimal dilution levels.

Conjugate Stability

HRP has good stability with covalent bonds to antibodies. The non-covalent bonds of biotin and avidin are very strong, thereby increasing sensitivity and stability. Biotin has a smaller molecular size compared to HRP. The conjugate is stored at 0 ℃ in 1X PBS buffer. Measurements of the biotin conjugate show better resistance and stability compared to the HRP conjugate in Table 4 and Table 5.

Table 4. Measurement of HRP Conjugate for Stability

Dillution	Absorbance (λ=450 nm)					
	1st Month		2nd Month		3rd Month	
	1	2	1	2	1	2
10^{-1}	2.015	2.102	2.186	2.134	0.280	0.267
10^{-2}	2.084	2.076	2.169	0.984	0.151	0.173
10^{-3}	2.006	2.112	1.930	0.020	0.106	0.104
10^{-4}	0.114	0.131	0.203	0.005	0.096	0.090
10^{-5}	0.009	0.014	0.011	0.000	0.084	0.083
10^{-6}	0.005	0.003	0.010	0.004	0.073	0.089
10^{-7}	0.002	0.002	0.014	-0.01	0.054	0.050

Table 5. Measurement of Biotin Conjugate for Stability

	Absorbance (λ=450 nm)							
*D	1st Month		2nd Month		3rd Month		4th Month	
	1	2	1	2	1	2	1	2
10^{-1}	2.13	2.16	2.14	2.13	2.13	2.12	0.14	0.16
10^{-2}	2.15	2.12	2.12	2.15	2.15	1.92	0.13	0.11
10^{-3}	2.14	2.15	2.13	2.13	2.14	2.08	0.08	0.11
10^{-4}	2.05	2.06	2.07	2.05	2.05	1.89	0.12	0.12
10^{-5}	0.48	0.52	0.56	0.49	0.48	0.32	0.07	0.08
10^{-6}	0.06	0.04	0.08	0.06	0.06	0.04	0.10	0.09
10^{-7}	0.05	0.03	0.06	0.04	0.05	0.03	0.052	0.053

*D=Dillution

Conjugates were stored at 0 ℃ in 1X PBS solvent. Measurements of the biotin conjugate (Table 5) had better durability and stability than the HRP conjugate (Table 4). This is due to the strong bond between biotin and avidin, which can increase the stability of the conjugate, making biotin conjugates more durable than HRP conjugates. The biotin and streptavidin complex helps to increase stability, as it has strong hydrogen bonds, as well as hydrophobic and van der Waals forces from the structure of biotin and streptavidin. Hydrogen bonds are bonds that occur between hydrogen atoms and negatively charged atoms that have free electron pairs (Dočkal et al., 2019). Van der Waals forces are interactions between molecules that form dipoles, thereby attracting other molecules momentarily (Feinberg et al., 1970). These forces and hydrogen bonds increase specificity or reduce non-specific binding in the ELISA system (Gao et al., 2025). The conformational effect also influences this stability; biotin will enter the streptavidin binding site and cover almost all of the biotin, thereby reducing dissociation from biotin and increasing stability.

Mehrotra et al. (2015) conducted stability research by storing conjugated antibodies without solvents. The conjugates were stored at temperatures ranging from 25 °C to 4 °C and -70 °C. The activity of the conjugates was then measured periodically during storage. This study concluded that 4°C was the best storage temperature and had the highest stability thus the selection for the storage temperature in this research is 0 ℃. HRP conjugates showed that the lower the concentration, the more unstable the conjugate, which could be due to several factors such as denaturation, oxidation, or others. Biotin conjugates have better stability; however, the factors affecting the storage of streptavidin-HRP need further investigation.

Conclusion

The antibodies were successfully conjugated with HRP and Biotin labels. The difference in molecular weight and size between the pre-conjugated antibodies and the conjugated antibodies was evident from the SDS-PAGE and colorimetry results, which proved the success of the conjugation. The optimal concentrations for HRP and Biotin conjugates are at dilutions of 10^{-3} and 10^{-4}. The stability of Biotin-labeled conjugates is higher than that of HRP-labeled conjugates under storage conditions of 0°C until the reading becomes unstable. The use of HRP conjugates is generally simpler than the use of biotin conjugates but has disadvantages in terms of specificity and sensitivity, while the use of biotin conjugates has very strong bond stability and better sensitivity but has a longer procedure compared to HRP conjugates.

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Author Contributions

NRF: Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. US: Conceptualization, Investigation, Methodology, Project Administration, Resources, Supervision, Writing – review & editing. HSD: Conceptualization, Funding

Acquisition, Methodology, Project Administration, Resources, Supervision, Writing – review & editing.

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Conflicts of Interest
The authors declare no conflict of interests.

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