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Original Article

Development of a sensitive, reliable, and cost-effective sandwich ELISA for quantitative measurement of human hemoglobin A2 in beta-thalassemia diagnosis

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Summary

Introduction: Accurate quantification of hemoglobin A2 (HbA2) is essential for the diagnosis and monitoring of beta-thalassemia and related hemoglobinopathies. Existing methods, such as HPLC and capillary electrophoresis (CE), are effective but require expensive instrumentation. We developed and validated a cost-effective and sensitive sandwich ELISA for measuring HbA2 levels in human blood samples.

Methods: A pair of specific monoclonal antibodies against distinct epitopes of HbA2 was used to establish a sandwich ELISA. The assay was optimized for analytical performance, including sensitivity, specificity, linearity, and intra-/inter-assay precision. Validation was conducted using both standard recombinant HbA2 and clinical samples from healthy donors and confirmed beta-thalassemia patients.

Findings: The assay demonstrated a lower limit of detection (LOD) of 0.1% and a quantification range of 0.1–10%. Coefficients of variation (CVs) were below 10% across all concentrations tested. HbA2 levels in beta-thalassemia patients were significantly elevated ($P<0.001$) compared to healthy controls, confirming the assay's diagnostic potential.

Conclusion: This optimized sandwich ELISA offers a reliable, affordable, and scalable alternative for quantifying HbA2 in clinical laboratories, particularly in resource-limited settings. Its high specificity and reproducibility make it a promising tool for the diagnosis and follow-up of beta-thalassemia.

Keywords: Hemoglobin A2, Sandwich ELISA, Beta-thalassemia, Immunodiagnostics, Biomarker quantification

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Introduction

Beta-thalassemia is a prevalent inherited blood disorder caused by mutations in the β-globin gene. These mutations result in reduced or absent production of β-globin chains, leading to ineffective hemoglobin synthesis and anemia.[1-3] This condition causes ineffective erythropoiesis and varying degrees of anemia, often requiring lifelong management.[4] One of the key biomarkers for the diagnosis of beta-thalassemia trait is hemoglobin A2 (HbA2), a minor component of adult hemoglobin composed of two α and two δ chains.[5,6] Elevated HbA2 levels, typically above 3.5%, are a key diagnostic marker for beta-thalassemia trait. Measurement of HbA2 is essential in carrier screening programs, genetic counseling, and the differential diagnosis of microcytic hypochromic anemia. However, it is important to consider that certain conditions, such as iron deficiency anemia, may influence HbA2 levels and complicate diagnosis. Therefore, HbA2 assessment should be interpreted alongside clinical and hematological parameters to ensure accurate identification of beta-thalassemia carriers.[7-9]

Currently, the gold standard for HbA2 quantification includeshigh-performanceliquidchromatography(HPLC) and capillary electrophoresis (CE).[10] These techniques provide high resolution and quantitative precision but suffer from significant limitations, especially in resource-limited settings.[11] The high cost of instrumentation, the requirement for trained personnel, and the lack of portability significantly restrict their use in decentralized laboratories and point-of-care testing environments.[12] Moreover, interference from hemoglobin variants (e.g., HbS, HbE) or post-translational modifications may affect the accuracy of these chromatographic methods in certain clinical contexts.[13]

To address these challenges, immunoassay-based methods, particularly enzyme-linked immunosorbent assays (ELISAs), have emerged as promising alternatives.[14] ELISAs offer several advantages: they are cost-effective, relatively easy to perform, adaptable to high-throughput formats, and require only minimal instrumentation.[15] Recent advances in monoclonal antibody production and assay design have further improved the sensitivity and specificity for detecting various hemoglobin subtypes, including HbF and HbS. However, data on the use of

ELISA for the quantification of HbA2 remain limited, and existing protocols often lack comprehensive analytical validation or demonstrated clinical application.[16]

In this study, we report on the development, optimization, and validation of a sandwich ELISA for the quantitative measurement of HbA2 in human blood samples. This method utilizes a pair of highly specific monoclonal antibodies targeting unique epitopes on the HbA2 molecule. The assay was systematically optimized for analytical performance metrics, including sensitivity, linearity, specificity, and reproducibility, and was validated using both recombinant standards and clinical specimens of individuals with beta-thalassemia as well as healthy controls.

By offering a simple, accurate, and affordable platform for HbA2 measurement, our proposed method addresses critical limitations of current diagnostic approaches and has potential applications in routine screening, epidemiological studies, and personalized patient monitoring, especially in low-resource and decentralized settings.

Methods

Reagents and materials

Carbonate-bicarbonate coating buffer (50 mM, pH 9.6), phosphate-buffered saline (PBS, pH 7.4), Tween-20, citrate-phosphate buffer (100 mM, pH 5.0), and sulfuric acid (2 M) were obtained from Merck (Germany). Bovine serum albumin (BSA), purified human HbA and HbA2 protein, and horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG were purchased from Sigma-Aldrich (USA). Two mouse monoclonal Antibodies against Human Hb/HbA2 (Rockland Capture mAb Pan-Hemoglobin (MV4) and detection mAb Anti-HbA2 specific (GS8) monoclonal antibody (mouse), Rockland Immunochemicals, Inc., USA).

Preparation of solutions

The composition, preparation details, and sources of all reagents and antibodies used in the sandwich ELISA are summarized in Table 1.

Coating of microplate

High-binding 96-well microplates (Nunc, Thermo Fisher) were coated with 100 µL/well of MAb1 diluted in carbonate-bicarbonate buffer to a final concentration of 5 µg/mL. Plates were sealed and incubated overnight at 4 °C.

Blocking

Following coating, wells were washed three times with PBST. Subsequently, 200 µL of blocking buffer was added to each well and incubated at room temperature (RT) for 2 hours. Plates were again washed three times before sample addition.

Table 1. Summary of analytical performance of the HbA2 sandwich ELISA

Parameter	Result
Specificity	No cross-reactivity with HbA, HbF, HbS, HbC
LOD	0.1% HbA2
LOQ	0.5% HbA2
Linearity	0.5%–10% ($R^2 = 0.996$)
Accuracy (vs. HPLC)	r = 0.961; bias = 0.23%
Intra-assay CV	2.4% – 4.8%
Inter-assay CV	3.2% – 6.1%
Clinical discrimination	$P < 0.0001$ (β-thalassemia vs. controls)

Preparation of conjugate

MAb2 was diluted in conjugate buffer to a concentration of 2 µg/mL and mixed with HRP-conjugated goat anti-mouse IgG (1:10 ratio). The mixture was incubated at RT for 1 hour with gentle agitation.

Standard preparation

A serial dilution of purified HbA2/HbA was prepared in blocking buffer to a final ratio of 0, 0.5, 1, 2, 5, and 10% and total Hemoglobin 1 mg/mL. A blank control (blocking buffer only) was included.

Assay procedure

After blocking, 100 µL of standards or test samples were added to the appropriate wells in duplicate. Subsequently, 100 µL of the prepared HRP-conjugated antibody solution was added to each well. Plates were incubated at RT for 1 hour and washed three times with PBST. Then, 100 µL of TMB substrate solution was added and incubated in the dark for 15 minutes at RT. The reaction was terminated by adding 50 µL of stop solution to each well. Absorbance was read at 450 nm using a microplate reader (BioTek, USA).

Data analysis

A standard curve was generated by plotting absorbance versus HbA2 concentration. Sample concentrations were interpolated from the standard curve using a four-parameter logistic regression model.

Analytical validation

- Specificity: Cross-reactivity with other hemoglobin isoforms (e.g., HbA, HbF, HbS) was assessed using purified proteins.
- Sensitivity: The limit of detection (LOD) and limit of quantification (LOQ) were determined based on signal-to-noise ratio (LOD = mean blank + 3 × SD-blank).
- Precision: Intra-assay and inter-assay variability were evaluated using replicate analysis (n = 6) of low, medium, and high HbA2 samples. Coefficients of variation (CV%) were calculated.
- Accuracy: The ELISA results were compared with

those obtained from HPLC (Bio-Rad Variant II) in 55 patient samples to assess method agreement (Pearson's correlation and Bland-Altman analysis).

Results

Specificity

The specificity of the developed sandwich ELISA for HbA2 was evaluated by testing potential cross-reactivity with structurally similar hemoglobin variants, including HbA, HbF, HbS, and HbC. No significant signal was observed with these variants, while a strong and consistent signal was obtained for HbA2. These results confirm that the assay exhibits high specificity for HbA2 and does not detect other hemoglobin types even at comparable concentrations.

Sensitivity and detection range

The analytical sensitivity of the assay was determined using serial dilutions of purified HbA2 standards. The lower LOD, calculated based on a signal-to-noise ratio of 3:1, was found to be 0.1% HbA2. The lower LOQ, defined as the lowest concentration with a coefficient of variation (CV) below 15% and accuracy within ± 15%, was established at 0.5%. The assay demonstrated a linear response between 0.5% and 10% HbA2, with a correlation coefficient (R^2) of 0.996.

Accuracy and method comparison

To assess the accuracy of the ELISA method, a total of 45 clinical samples (25 β-thalassemia carriers and 20 healthy controls) were analyzed using both the sandwich ELISA and the reference HPLC method. A strong positive correlation was observed between the two methods (Pearson correlation coefficient $r = 0.961$, $P < 0.001$). Bland–Altman analysis showed a mean bias of 0.23%, with limits of agreement ranging from –0.85% to + 1.31%, indicating minimal systematic deviation and acceptable agreement between the methods.

Precision (repeatability and reproducibility)

The precision of the assay was evaluated through intra-assay and inter-assay measurements. Intra-assay precision was assessed by analyzing three concentration levels (0.5%, 3%, and 7% HbA2) in six replicates within a single run, yielding CVs of 2.4%, 3.1%, and 4.8%, respectively. Inter-assay precision was determined by analyzing the same samples across three independent runs on different days, resulting in CVs ranging from 3.2% to 6.1%. These results confirm that the assay demonstrates high repeatability and reproducibility, following accepted bioanalytical guidelines.

Clinical sample application

The clinical applicability of the assay was validated by comparing HbA2 levels in patient and control samples. The mean HbA2 concentration in β-thalassemia carriers was 5.4% ± 0.6%, while in healthy individuals it was 2.1% ± 0.4%. The difference between the two groups was statistically significant (unpaired t-test, $P < 0.001$), indicating the assay's potential utility in clinical screening and diagnosis of β-thalassemia trait.

Standard curve

As showed in Figure 1, the standard curve was generated by plotting the optical absorbance (OD) at 450 nm against known HbA2 concentrations (up to 10%). A strong linear relationship ($R^2 > 0.996$) was observed.

Summary of assay performance

A summary of the analytical performance characteristics of the sandwich ELISA for HbA2 is presented in Table 2.

Discussion

Quantitative measurement of HbA2 plays a critical role in the diagnosis and management of beta-thalassemia, as well as other hematologic disorders characterized by abnormal globin chain expression. The current gold standard for HbA2 quantification is HPLC, a method with well-established accuracy and resolution.[17,18] However, HPLC has significant limitations, particularly in low-resource settings, due to its dependence on expensive instrumentation, specialized training, and complex maintenance requirements. Alternative techniques, such as electrophoresis and capillary zone electrophoresis, also pose similar challenges and are not easily scalable for population-based screening programs.[19,20]

In this study, we developed and analytically validated a sandwich ELISA-based method for the detection and quantification of HbA2 in human samples. The assay utilizes two monoclonal antibodies that selectively bind to distinct epitopes on the HbA2 molecule, enabling

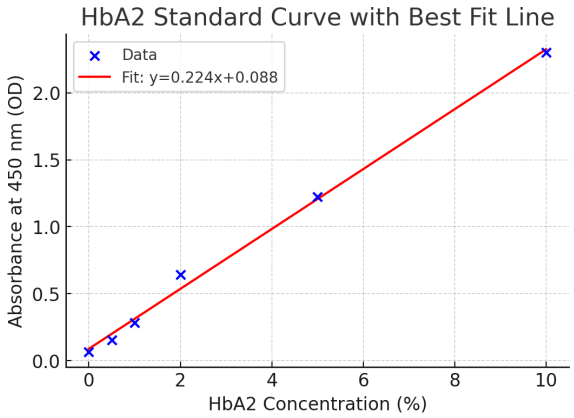

Figure 1. Standard curve for HbA2 sandwich ELISA. The standard curve was generated by plotting the absorbance values at 450 nm against known HbA2 concentrations ranging from 0% to 10%. A strong linear relationship ($R^2 > 0.996$) was observed, indicating acceptable assay linearity and a proportional increase in signal intensity with increasing HbA2 concentration. This standard curve was used to calculate the HbA2 concentrations in unknown samples by interpolation

Table 2. Standard HbA2 concentrations and corresponding absorbance values at 450 nm

HbA2 concentration (%)	Absorbance (450 nm)
0	0.064
0.5	0.154
1	0.282
2	0.643
5	1.223
10	2.302

robust and specific detection. Our data demonstrated that this immunoassay exhibits high analytical performance across key parameters, including specificity, sensitivity, accuracy, and precision.

Specificity was established by evaluating cross-reactivity with common hemoglobin variants (HbA, HbF, HbS, and HbC), none of which showed interference in the assay, supporting its molecular selectivity. The assay also demonstrated excellent analytical sensitivity, with a LOD of 0.1% and a LOQ of 0.5% HbA2, which are clinically relevant thresholds for thalassemia diagnostics. Furthermore, comparative analysis with HPLC on a clinical laboratory sample revealed a high degree of agreement between the two methods (Pearson's $r = 0.96$, $P < 0.001$), and Bland–Altman analysis confirmed that the deviation between methods remained within acceptable clinical limits. Precision studies indicated low intra-assay and inter-assay coefficients of variation, further supporting the assay's reproducibility.

The operational advantages of this ELISA approach are noteworthy. The method is cost-effective, requires no advanced instrumentation, and is amenable to standard laboratory workflows. It also lends itself well to high-throughput implementation, making it particularly valuable in regions with a high burden of hemoglobinopathies but limited diagnostic infrastructure.

Despite these advantages, a key limitation of the proposed method lies in its dependence on high-quality monoclonal antibodies, which can be costly to produce or procure. However, once established, the per-sample cost of ELISA remains substantially lower than HPLC method, especially when deployed at scale. Moreover, the standardized nature of the protocol—including detailed procedures for coating, blocking, antibody conjugation, and calibration—facilitates reproducibility and transferability across laboratories.

Conclusion

We present a novel, validated sandwich ELISA method for the quantitative determination of HbA2 in human blood samples. This assay demonstrates acceptable specificity, sensitivity, accuracy, and reproducibility, and offers a practical alternative to the HPLC methods. Its simplicity and affordability make it an attractive tool for laboratories in low- and middle-income countries, and its adaptability for high-throughput testing positions it as a valuable resource for public health screening programs targeting beta-thalassemia carriers and patients.

The method has the potential to improve early detection, diagnosis, and monitoring of hemoglobinopathies, particularly in underserved settings where conventional chromatographic platforms are inaccessible. Furthermore, the detailed standardization of assay materials and procedures enhances its utility for research and clinical implementation alike.

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Artificial Intelligence Use Disclosure

None.

Authors' Contribution

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Writing-review & editing: Safura Pakizehkar and Mehdi Hedayati.

Competing Interests

The authors declare that there is no conflict of interest related to this study. This research was conducted independently and was not supported or influenced by any commercial or non-commercial entities. All reagents and materials used were procured from standard commercial suppliers, as detailed in the Methods section. The authors have no financial or institutional affiliation that may be perceived as a potential conflict regarding the findings or interpretation of this work.

Ethical Approval

In vitro method development approved by National Institute for Medical Research Development (NIMAD).

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