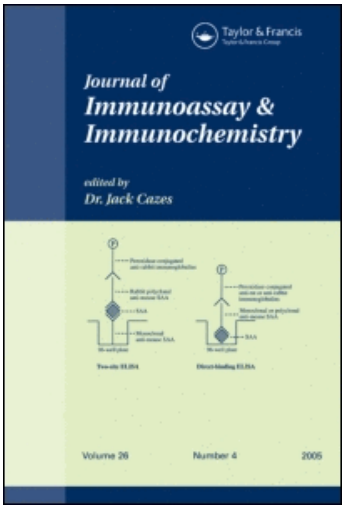


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# Development and Validation of an ELISA for Hemoglobin-$A_2$: A Novel Method for β-Thalassemia Screening in Developing Countries

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## ABSTRACT

Hemoglobin-$A_2$ ($HbA_2$) measurement in human hemolysates has great significance, since its level can indicate $\beta$-thalassemia carrier status in otherwise healthy individuals. An ELISA for $HbA_2$ using antiserum monospecific to the $\delta$ chain of $HbA_2$ and affinity purified antirabbit gamma globulins (ARGG) conjugated to horseradish peroxidase (HRP) have been developed. The monospecific antiserum used does not cross react with other hemoglobins. Hemolysates from volunteers are used for measure-

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ment of $HbA_2$. In a limited trial for $\beta$ -thalassemia carrier screening ($n = 350$), the results obtained with the developed ELISA are comparable with those obtained with a micro-column chromatography method ($r \geq 0.89$). The developed ELISA is simple, accurate, precise, inexpensive, and several samples can be processed simultaneously with ease, making this system a suitable candidate for transforming into a user friendly kit.

*Key Words:* Hemoglobin-$A_2$; ELISA; $\beta$-Thalassemia; Hemolysates.

## INTRODUCTION

The hemoglobinopathies, or hemoglobin disorders, are probably the most common single genetic category of diseases in the world population. These form a major group with an estimate of $250 \times 10^6$ individuals with this condition throughout the world (4.5% of the total world population).[1] The thalassemias, the commonest monogenic diseases, are a family of inherited disorders of hemoglobin synthesis characterized by a reduced output of one or other of the globin chains ($\alpha$ or $\beta$) of adult hemoglobin, which results from markedly decreased amounts of globin messenger ribonucleic acid. The imbalance may result from many genetic lesions. They are likely to pose an increasing health problem for many developing countries during the early part of the new millennium.

The thalassemia syndromes are classified, according to the particular globin chain that is ineffectively produced, as $\alpha$, $\beta$, $\delta\beta$ and $\gamma\delta\beta$ thalassemia. The $\alpha$ and $\beta$ thalassaemia are by far the most important. Because of the lack of knowledge in the general population in India, the frequency of $\beta$-thalassemia ranges from 3.5% to 15%.[2] Every year about 100,000 children with thalassemia major are born, of which nearly 10,000 are in India, constituting 10% of the total number in the world.[2] Many studies carried out amongst a mixed population in Mumbai, India, revealed that the percentage of thalassemia carriers is as high as 3.5–4%, and this is corroborated by two Indian Council of Medical Research initiated studies carried out by our group.[3–5]

In normal individuals, the mean proportion of $HbA_2$ is 2.5% of total hemoglobin, whereas that for $\beta$-thalassemia heterozygotes is high and varies from 3.7% to 7.0%. This abnormally high concentration of ($HbA_2$) is a generally accepted principal diagnostic feature and is generally estimated by ion-exchange chromatography or cellulose acetate electrophoresis. The results obtained from these methods are not reproducible and techniques require well-trained personnel.

Making use of the difference in structures of $\beta$ and $\delta$ chains of HbA and $HbA_2$ respectively, to raise antiserum specific for the $\delta$-chain and employ it for





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an immunological assay (ELISA) to quantitative $HbA_2$ is the basic concept of this study. This is a novel approach and may be the first initiative of this kind in India. This technique requires the incorporation of monospecific antisera capable of specifically reacting only with $HbA_2$, i.e., with the $\delta$-chain of $HbA_2$. The ELISA technique is more sensitive and specific than the biochemical methods used currently.[6] The ELISA can be employed either in conjunction with routine methods of $\beta$-thalassemia screening (blood cell indices, naked eye single tube red cell osmotic fragility test (NESTROFT), etc) or can be used alone to screen populations. Qualitative or quantitative ELISAs may be used to suit our requirement, eliminating the use of expensive and time-consuming techniques. Mass screening programs to detect carrier status have been done successfully in many countries. In Cyprus with an excellent tradition of preventive medicine, involving population screening, genetic counseling along with prenatal diagnostic facilities for "at risk" couples for thalassemia, could eradicate the disease completely.[7] In developed countries, the self-contained and well-simplified ion-exchange chromatography kits are available and being routinely used. Perhaps this might have prevented the development of ELISA based diagnostic kits for screening of thalassemia. However, immunological assays for various hemoglobins have been successfully developed and used in the USA.[8,9]

Unfortunately development of cost effective and simpler immunological tests for $HbA_2$ have yet not been undertaken in India. Most of the mass screening programs make use of the electrophoresis or micro-column chromatography procedures, which require the samples to be transported to well-equipped laboratories, rendering mass screening programmes impossible in rural hospitals. The project aims at developing the technology and ensuring its utility in the control of thalassaemia. The success of the project and dissemination of technology will pave a path for other workers to change over from laborious or expensive traditional assays.

## EXPERIMENTAL

### Isolation and Purification of $HbA_2$ and HbA

$HbA_2$ and HbA were isolated from erythrocytes of normal adult volunteers. RBC of 20–30 mL were washed thrice in physiological saline and lysed by adding one volume of water and two volumes of carbon tetra chloride. After vigorous shaking in a mechanical agitator, the mixture was centrifuged at 3000 rpm for 20 min. The hemoglobin solution, which settles on top, is the hemolysate and was subjected to ion-exchanger chromatography on preparative columns (30 cm $\times$ 4 cm) using DEAE cellulose (Whatman DE-52). In Tris–HCl buffer,





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50 mM/L $HbA_2$ is eluted out at pH 8.3, and HbA at pH 7.0. The purified fractions were dialyzed against 0.15 M NaCl and lyophilized; single band on cellulose acetate paper; electrophoresis confirmed the purity of the fractions.[10]

## The Development of Monospecific Antiserum to δ Chain of $Hba_2$

Two adult rabbits (SHL breed) were immunized with 20 mg of purified $HbA_2$ in 1 mL of 0.15 M NaCl (saline), emulsified with an equal volume of Freund's complete adjuvant (FCA-Sigma Chemicals, USA) subcutaneously, on multiple sites on the back of the animal. A booster injection of 10 mg of protein dissolved in 1 mL of saline and emulsified in 1 mL of Freund's incomplete adjuvant (FIA) was administered 4 weeks later. Antibody titer was checked weekly by immunodiffusion and later by a simple ELISA procedure. Four weeks later, a second dose of booster was administered (quantity and volume the same as the first booster).[8] The developed antisera showed strong immuno-reactivity with $HbA_2$ and weak immuno-reactivity with HbA in a immunodiffusion test. The $HbA_2$ antiserum was rendered monospecific to the δ chain of $HbA_2$ by solid phase immuno-adsorption with HbA and plasma insolubilized and polymerized with glutaraldehyde.[11] In brief, human plasma (100 mL) and HbA (1 g) were mixed with 10 mL of 2 M/L acetate buffer pH 5, and 30 mL of 2.5% v/v glutaraldehyde was added to this mixture, drop wise. The mixture was allowed to stand for 2 hr and formed gel, and was then dispersed in 500 mL of 100 mM/L phosphate buffer pH 7.4 and homogenized. The homogenate was then centrifuged at 4000 rpm for 15 min and the supernatant was discarded. The gel was then dispersed in 300 mL of 100 mM/L phosphate buffer, pH 7.4 and centrifuged at 4000 rpm. This was repeated till the O.D of the supernatant was less than 0.050 at 280 nm. The gel was preserved at 4°C until it was used. The gel was dried on filter paper and was added to the neat $HbA_2$ antiserum to cover it completely. It was left at room temperature for 1 hr and later at 4°C overnight. The antiserum with the gel was then centrifuged at 3000 rpm to recover the monospecific antiserum. This antiserum, after adsorption, showed no immuno-reactivity with HbA in immunodiffusion studies.

## Purification of Antirabbit IgG by Affinity Chromatography

Rabbit serum IgG was purified by ion-exchange chromatography on DEAE cellulose as described by Huisman and Dozy (1965),[10] and two goats were immunized with the same. The rabbit IgG was conjugated to cyanogen bromide activated sepharose (Pharmacia, Sweden). IgG-sepharose,





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(5 mL) was incubated overnight with about 100 mg of gamma globulins from goat antiserum to rabbit IgG, at 4°C with occasional gentle shaking. Next day, the unbound proteins were washed off with 100 mmol/L phosphate buffer pH 7.4 containing 0.5 M NaCl on a sintered glass funnel, and the bound antibody was eluted with 3 M/L NaSCN, dialyzed against distilled water overnight with three changes of 100 mM/L phosphate buffered saline, pH 7.4, and concentrated by lyophilization.

This purified fraction showed very high immuno-reactivity with normal rabbit gamma globulins and was used for conjugating to horseradish peroxidase (HRP).[12]

## Conjugation of Peroxidase to ARGG Using NHS-Ester-Maleimide-Mediated Reaction

Heterobifunctional reagents containing an amine reactive NHS ester on one end and a sulfhydryl-reactive maleimide group on other end, generally have a great utility for producing antibody–enzyme conjugates. Cross linking reagents possessing these functional groups can be used in highly controlled multiuse procedures that yield conjugates of defined composition and high activity.

Succinimidyl-4-(*N*-maleimidomethyl)-cyclohexane-1-carboxylate (SMCC) (Pierce, Rockville, IL, USA) was the cross linker used in preparation of antibody–enzyme conjugates. The SMCC activated HRP was then conjugated to antirabbit gamma globulins (ARGG) with reduced disulfide groups with 2-mercapto ethylamine (Pierce).[13]

In brief, 40 mg of HRP (Sigma Chemical) was dissolved in 2 mL of 100 mmol/L sodium phosphate, containing 0.15 M NaCl, pH 7.2. Sulfo-SMCC (6 mg) was then added to this solution, mixed and kept for 1 hr at room temperature, and then purified by gel filtration on Sephadex G-25. HRP concentration was adjusted to 10 mg/mL and immediately used to conjugate with ARGG. ARGG solution (8 mg/mL), (2 mL), was first reduced by adding 12 mg of 2-mercapto ethylamine for 90 min at 37°C, then purified on sephadex G-25. This reduced ARGG (1.9 mL) was then mixed with 2 mL of enzyme solution and allowed to react for 30–60 min at 37°C. The conjugate was frozen and then stored.

## The Standardization of ELISA

The procedure used was a direct ELISA, essentially similar to that described by Graver (1984), with suitable modifications required for the





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enzyme HRP. The wells of polystyrene microtiter plates (Nunc modules) were adsorbed with 200 μL of adsorption buffer (100 mmol/L bicarbonate buffer pH 9.6.) containing 125 μg of $HbA_2$ per mL of buffer. After overnight incubation at 4°C, the plates were washed three times with 100 mmol/L phosphate buffer, pH 7.2 containing 0.05% tween-20 (PBST). Unoccupied sites were blocked by adding 200 μL of 1% w/v bovine serum albumin, in 100 mMol/L PBS, pH 7.2 to each well, and incubated at R.T. for 1 hr, followed by 3 washes in PBST.

For quantitative analysis, dose response three curves were developed by preparing varying concentrations of $HbA_2$ in HbA, ranging from 0% to 10% of $HbA_2$ in HbA, and the final concentrations to 50 μg of total Hb/mL. This was achieved by preparing a stock solution of HbA (500 μg/mL) and $HbA_2$ (50 μg/mL) and combining them in varying proportions to get the required percentages with final protein concentration constant at 50 μg/mL. The sample hemolysates were also diluted accordingly to 50 μg/mL with PBST. To each well of microtitration plate, 150 μL of anti-$HbA_2$ diluted 1 : 1000 in PBST was dispensed. At the end of 90 min incubation at 37°C, the wells were rinsed three times with PBST.

Then, 200 μL of 1 : 5000 diluted ARGG–HRP conjugate was dispensed. After 1 hr at 37°C, wells were washed three times with PBST, followed by the addition of 200 μL of *o*-phenylenediamine (OPD) in citrate buffer containing 0.5 μL of $H_2O_2$. The reaction was terminated by the addition of 4 N $H_2SO_4$ after 20 min. Absorbance was measured at 490 nm on an ELISA reader (Biotech USA). All analyses were performed in duplicate, and results were expressed as the mean of these determinations.

To 1 mL aliquot of a hemolysate of known $HbA_2$ concentration (2%), varying amounts of $HbA_2$ standard was added (+2%, +4%, +8%) and measured by ELISA.

For the precision of the assay, replicates of two internal quality control pools (one normal and the second from a thalassemia carrier) were assayed for the determination of intra-assay and inter-assay variations.

$HbA_2$ was also measured by micro-column chromatography (Bio-Rad, USA), as per the instructions of the manufacturer, and the results were compared with that of ELISA.

## RESULTS

Ion-exchange chromatography using DEAE-cellulose on preparative columns, yielded $HbA_2$ fractions at pH 8.3 and HbA at pH 7. These fractions on cellulose acetate electrophoresis at pH 8.9, showed single bands with





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electrophoretic mobility similar to the reference $HbA_2$ and HbA. This purified $HbA_2$ was used for immunizing two adult rabbits.

The antiserum raised showed immuno-reactivity with $HbA_2$ but, also, mildly cross-reacted with HbA. After adsorption with HbA, the $HbA_2$ antiserum was made monospecific. Further evidence of monospecificity of the antiserum is shown by inhibition assays where hemoglobins A, S, and F were ineffective in blocking the binding reaction. While the percent of inhibition by $HbA_2$ was 80% at 100 $\mu$g/mL, it varied between 24 and 26% with other hemoglobins. This well indicates the monospecificity of the developed antiserum to the $\delta$ chain of $HbA_2$.

Affinity purified ARGG was used for conjugating to HRP using the NHS-ester-maleimide-mediated method. The conjugate thus obtained had an optimum working dilution of 1 : 5000.

A 1000 fold dilution of anti-$HbA_2$ yielded an absorbance of 1.8 at 492 nm and was used in subsequent experiments. The polystyrene plates were adsorbed with 125 $\mu$g/mL of $HbA_2$, as it was shown that a concentration of 25 $\mu$g/well was enough to saturate them maximally.[8]

The sensitivity of the ELISA was adjusted to accommodate a physiological concentration of $HbA_2$ (0.8%). For quantitative measurement of $HbA_2$, a standard dose–response curve was then devised from hemoglobin mixtures, which were prepared by combining known amounts of $HbA_2$with HbA ranging from 0% to 10% $HbA_2$. As shown in the standard curve, $HbA_2$ quantities ranging from 1% to 10% fell on the linear portion of the curve and this range was used to quantify $HbA_2$ in hemolysates. Each point on the curve represents the mean of 12 individual determinations performed in duplicates (Fig. 1).

The sample dilution curve was found to be parallel to the standard curve, both in normal, as well as in thalassemia carriers, indicating no sample interference (Fig. 2).

In recovery experiments, the percent of recovery varied between 98% and 103.2%, well within acceptable ranges. (Table 1)

The cross reactivity studies showed that hemoglobins A, S, and F were ineffective in displacing $HbA_2$. While the percent of inhibition by $HbA_2$ was 80% at 100 $\mu$g/mL, it varied between 24% and 26% with other hemoglobins.

The precision of the developed assay was good, as indicated by low inter-assay and intra-assay variations of quality control pools (Table 2).

A good correlation ($r = 0.9$) is observed between the quantities of $HbA_2$ in hemolysates from normal and thalassemia carrier individuals, as measured by both the developed ELISA and standard micro-column chromatography (Fig. 3).





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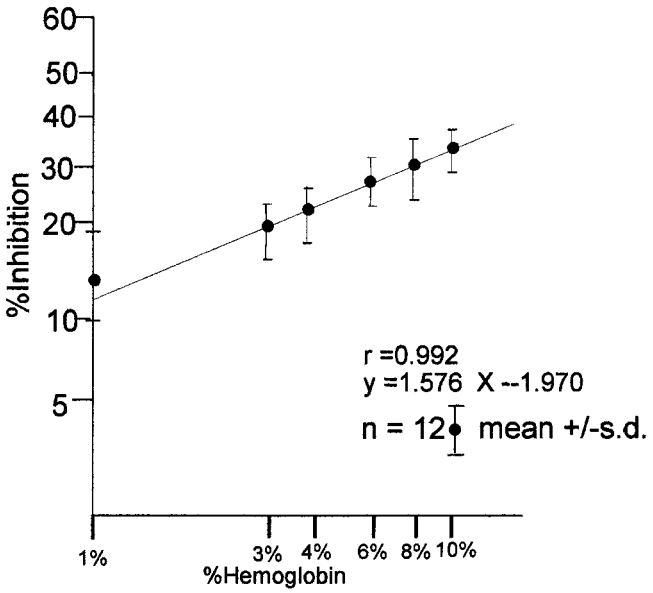


***Figure 1.*** Standard curve.

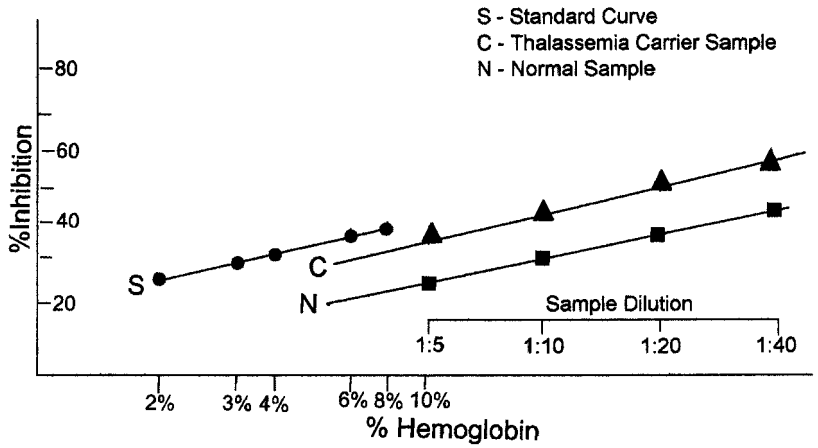


***Figure 2.*** Sample dilution curves.





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***Table 1.*** Recovery of $HbA_2$ from standard hemolysate samples.

| Amount added to hemolysate, % | Amount recovered | Percentage recovered |
|---|---|---|
| +2 | 1.99 | 98.7 |
| +4 | 4.13 | 103.16 |
| +8 | 7.95 | 99.5 |

### Prediction of β-Thalassemia Carrier Status in Screening Programmes

Hemolysates from 350 subjects were collected and analyzed for their $\beta$-thalassemia status.

The $HbA_2$ values ranged between 1.5% and 3.2% (mean = 2.5% ± SD 0.64) in normal individuals by micro-column chromatography and 1.2 and 3.5 (mean = 2.4% ± SD 0.6) by ELISA. In thalassemia carriers $HbA_2$ values ranged between 3.8% and 5% by ELISA and 3.6% and 4.5% by micro-column chromatography (Fig. 4).

## DISCUSSION

Laboratory diagnosis of $\beta$-thalassemia traits primarily depends upon the quantitation of $HbA_2$ in adult hemolysates. For screening purposes, the most widely accepted technique is micro-column chromatography.[14] The immunological methods are less expensive than the micro-column technique, but also, more specific, sensitive, and reproducible.

Cellulose acetate electrophoresis is used to separate HbA and $HbA_2$. At pH 8.9, $HbA_2$ is separated from HbA and eluted into the buffer.

***Table 2.*** Intra- and inter-assay coefficient of variation in $HbA_2$ measurements in two quality control pools.

| | | Coefficient of variation in % | |
|---|---|---|---|
| Pool | Mean value (%$HbA_2$) ± SD | Intra-assay ($n$ = 10) | Inter-assay ($n$ = 10) |
| 1 (Normal) | 2.47 ± 0.12 | 3.5 | 4.85 |
| 2 ($\beta$-Thalassaemia carrier) | 4.14 ± 0.33 | 4.7 | 7.97 |





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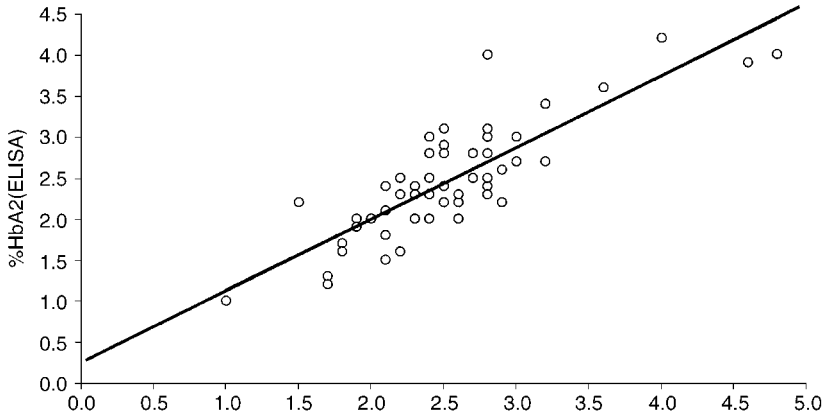


***Figure 3.*** Comparison of HbA2 values as estimated by ELISA with micro-column chromatography.

The absorbance of the eluent was measured and compared with that of remaining hemoglobin to calculate percentage.[15] This method is unsuitable in the presence of hemoglobins with electrophoretic mobility similar to that of $HbA_2$. Therefore, a test, which is more specific and yields quantitative results faster, would be of greater assistance in establishing the correct diagnosis.

Over the next decade, it will be essential to make the thalassemia problem more visible to governments and international health agencies that are involved in health care in emerging countries. The partial control of the disease by carrier detection and prenatal diagnosis, will only be feasible in

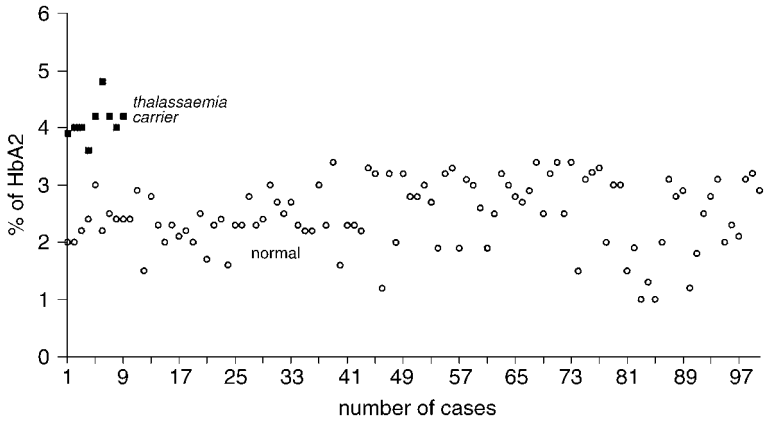


***Figure 4.*** Percent HbA2 as measured by ELISA.





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emerging countries if it is possible to obtain the financial support of the major international agencies and the cooperation of their governments and communities; the remarkable success of this approach in some of the Mediterranean islands is a good example of what can be achieved. A very sensitive assay could be significant in detecting small amounts of $HbA_2$ in hemolysates and could replace the more expensive and tedious methods, especially in large screening programmes in our country.

Considering the similarity in structures of various hemoglobins present in the sample, raising specific monoclonal antibodies to $HbA_2$ will be the answer to eliminate cross-reactivity. However, this approach is very expensive. In the present study by adsorption of antiserum with HbA, we are successful in removing cross-reactivity with HbA and rendering the antiserum monospecific to the $\delta$ chain of $HbA_2$ and achieving an identical degree of specificity at reasonably low cost.

In the initial phase of the project, we have successfully developed an ELISA, which has great potential in the control of thalassemia in developing countries like India. Conceivably, this technique could be adapted for use in mass screening programmes for $\beta$-thalassemia carriers. Moreover, the success of the project and dissemination of technology will pave the path for other workers to change over from laborious or expensive traditional assays.

## CONCLUSION

The study shows that the developed ELISA is simple to perform, cost effective, and has potential to be used in screening programmes for $\beta$-thalassemia carriers.

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