A microplate reader-based method to quantify NADH-cytochrome b5 reductase activity for diagnosis of recessive congenital methaemoglobinemia

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ABSTRACT
Objectives: Congenital methemoglobinemia due to NADH-cytochrome b5 reductase 3 (CYB5R3) deficiencies is an autosomal recessive disorder that occurs sporadically worldwide. A sensitive, accurate, and rapid analysis of NADH-CYB5R enzyme concentrations is necessary for the diagnosis of RCM. Here we present an alternative microplate method that is based on a standard 96-well microplate format and microplate reader that simplify the quantification of NADH-CYB5R activity.

Methods: TECAN (Infinite 200 PRO series) microplate reader with Tecan’s proven Magellan™ software measured the NADH-CYB5R enzyme activity in 250 normal controls and previously diagnosed 25 cases of RCM due to NADH-CYB5R deficiency in the Indian population using 96-well microplates using 200 μl of total reaction mixture and also compared with standard spectrophotometric assay. We have also studied stability of the hemolysate stored at 4 and −20°C temperature.

Results and discussion: Enzyme activity in all 25 samples ranged from 6.09 to 10.07 IU/g Hb (mean ± SD: 8.08 ± 1.99 IU/g Hb) where as normal control ranged (n = 250) between 13.42 and 21.58 IU/g Hb (mean ± SD: 17.5 ± 4.08 IU/g of Hb). Data obtained from the microplate reader were compared with standard spectrophotometer method and found 100% concordance using both methods. Microplate method allows differentiating between normal, deficient and intermediate enzyme activity. It was observed that samples had significant loss of activity when stored at 4°C and retained stable activity at −20°C for 1 week time.

Conclusion: Our new method, incorporating a whole process of enzyme assay into a microplate format is readily applicable and allows rapid monitoring of enzyme assay. It is readily applicable to quantitative assay on pediatric sample as well as large number of samples for population screening.

KEYWORDS
Microplate reader; NADH-cytochrome b5 reductase deficiency; recessive congenital methaemoglobinemia

Introductions
Recessive congenital methemoglobinemia (RCM) is a relatively rare clinical disorder characterized by lifelong cyanosis, due to deficiency of NADH-cytochrome b5 reductase (NADH-CYB5R) [1]. Diagnosis of RCM involves clinical signs and symptoms, and family history of patient. A sensitive, accurate, and rapid analysis of NADH-CYB5R enzyme concentrations is necessary for the diagnosis of RCM and many laboratory approaches have been developed for this purpose. The earliest method measures the diaphorase activity of b5R, in which 2, 6-di-chlorophenol-indo-phenol (DCIP) was used as an artificial substrate in the presence of NADH. This was followed by two procedures devised by employing the methemoglobin reductase activity of the enzyme [2–4]. It was not until the discovery of the natural substrate of CYB5R, cytochrome b5R that assays based on the use of cytochrome b5 was established [5]. Also, a method for the electrophoretic visualization of the NADH-CYB5R band in gels was described by Kaplan and Beutler, using the substrate 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT), together with NADH and DCIP [6]. To establish a diagnosis of RCM, it is important to demonstrate quantization of enzyme deficiency of NADH-CYB5R and this is usually done using a spectrophotometric method. This assay is based on the decrease in the absorbance of the substrate NADH, which is used up by the enzyme to reduce ferrihemoglobin. The absorbance is measured at 340 nm. This decrease in the absorbance is used for measurement of the enzyme activity [7]. Another qualitative fluorescence spot test is used for the detection of NADH-CYB5R activity in RBC hemolysate and was developed by Feng Hua-Lan et al., based on recombinant human erythrocyte b5R monoclonal antibodies [8]. This test is sensitive and reliable but most expensive. The complexity of these methods and their relatively large sample size requirements (generally at least 5 ml) and it has limited use and inability to diagnose of NADH-CYB5R activity in the pediatric samples.

Microplate-readers are instruments designed to measure the absorbance, fluorescence, or luminescence of samples in microtiter plates. The plates...
typically consist of 96 wells, with the maximum volume of 360 μl per well. Their main advantages are small sample volume, high-throughput of samples and easy to use. They have been widely used in immunological assay for many years [9], but their use in enzyme kinetic study is limited. In this paper we describe the development of a microplate-plate based assay to detect NADH-CYB5R activity by fluorescence and absorbance measurement in the 200 μl of reaction mixture containing NADH, which is a simple, low-volume, and low-cost method for NADH-CYB5R quantization. It will be useful in neonatal samples and very useful for population screening.

Materials and methods

Clinical protocol and characteristic of patients

At the National Institute of Immunohematology (NIIH) Mumbai, we have previously diagnosed 25 cases of RCM due to NADH-CYB5R deficiency in the Indian population using standard method. Patient’s data regarding hematological, biochemical, and molecular characterization were published previously [10]. These 25 patients were diagnosed based on the history of cyanosis, bluish discolouration of nail beads and tongue due to increased level of methemoglobin associated with NADH-CYB5R deficiency by standard method. In all the cases, the level of methemoglobin has increased varying from 5.80–40.52% (normal range : <2.0%) and reduced NADH-CYB5R activity are summarized in Table 1. Control group included 250 healthy adult donors, who were randomly selected blood bank donors. Two milliliters of blood samples were collected in ethylene diamine tetra acetic acid (EDTA). Written informed consent was obtained from all patients after a full explanation of the study protocol related to RCM disease. The present study was approved by the ethical committee of the NIIGH Mumbai. The enzyme activity was analyzed by both microplate as well as standard spectrophotometric method.

Analytical method

This work used a TECAN Infinite 200 PRO series (Manedorf, Switzerland) microplate reader equipped with software version V6.5 Magellan™. The Infinite 200 PRO can provide a full range of leading detection methods in one easy-to-use modular instrument, with either Quad4 monochromator™ (Infinite M 200 PRO) or filter-based technologies (Infinite F200 PRO). Tecan’s proven Magellan™ software provides features that perfectly match the flexibility of the Infinite 200 PRO. The analyzer was calibrated, controlled, and maintained according to the manufacturer’s recommendations.

Preparation of hemolysate

Two milliliter of whole blood was collected after informed consent from a healthy adult (regular blood donor), type I and II RCM patients previously diagnosed and their relatives. Red blood cells were separated and three times washed with normal saline (0.9% NaCl) and 5 μl of packed red cells were lysed by adding 95 μl of stabilizing solution (10 mM Tris–Cl (pH 7.5), 1 mM EDTA, 10 mM dithiothreitol (DTT)), followed by repeated freezing and thawing. Centrifugation at 12 000g for 20 minutes was carried out and the supernatants recovered and used as a sample for the enzyme assay. Hemoglobin was estimated by Drabkin’s method in hemolysate. Activity thus determined was expressed as International units per gram hemoglobin (IU/g Hb).

Reaction mixture for enzyme assay procedure

NADH-CYB5R activity was measured by the standard method described [11]. Reagent 1 consists of 100 μl of 0.1 M Tris–HCl (pH 7.5), 100 μl of 2 mM NADH (Sigma, Aldrich), and 690 μl of purified Milli-Q water. For convenience, all the above components were mixed in suitable proportion and 178 μl of this mixture was added to each well of the microtiter plate (Corning™ Costar™ 96-Well Plates, Thermo fisher Scientific, Pittsburgh, PA, U.S.A). After this, the plate was incubated at 37°C for 10 minutes. Reagent 2 consist of 200 μl of 2 mM potassium ferricyanide and 20 μl of hemolysate (1:20 diluted) is mixed 1.5-ml glass tube. From this mixture, 22 μl is taken and mixed with 178 μl of incubated reagent 1 (for TEST, and not for BLANK) in the microplate well. Readings were taken immediately at 340 nm. The program for this entire kinetic assay, i.e. from incubation up to taking readings was created using the software MAGELLAN V6.5 in the TECAN Infinite 200 PRO microplate reader. Decrease in the absorbance of NADH at 340 nm was observed for 10 minutes and delta OD (ΔO.D) is used for measurement of the activity of the NADH-CYB5R. Conversion of NADH to NAD was assayed by monitoring decreased in absorbance at 340 nm. One unit of enzyme activity was defined as 1 mmol of NAD produced per minute.

Enzyme activity is calculated by using following formula:

\[
\text{Enzyme activity(Unit/ml)[A] = \frac{\Delta O.D}{\varepsilon \times N \times V_{ml}}}
\]

where \(\varepsilon\) = millimolar extinction coefficient for NADH/ NAD system is 6.22; \(N\) = the number of molecules of NADH converted per mole of substrate consumed (\(N = 1\)); \(V_{ml}\) = The volume of hemolysate added to the cuvette in ml; \(\Delta O.D\) = the change in optical density (absorbance) per minute.
Table 1. Showing meth-Hb level, comparison between spectrophotometer and 96-well plate method of NADH-cytochrome b5 reductase activity in fresh hemolysate store for 1 week and 2 weeks at two
different temperatures 4 and −20°C and their genotypes in 25 RCM patients.

| SL. No. | Case ID | Age/sex | Meth-Hb level (%) | NADH-Cyb5r activity in fresh sample (IU/g Hb) | NADH-Cyb5r activity after 1 week (IU/g Hb) | NADH-Cyb5r activity after 2 week (IU/g Hb) | Genotypes |
|--------|---------|---------|-------------------|---------------------------------------------|-------------------------------------------|------------------------------------------|------------|
|        |         |         |                   | Spectrophotometer | 96-well plate method | 4°C | −20°C | 4°C | −20°C | |
| 1      | BS      | 13 Y/M  | 23                | 21.43            | 6.80             | 4.40 | 6.40 | 2.50 | 5.10 | Gly154Glu (Homo) |
| 2      | SM      | 20 Y/M  | 18                | 24.64            | 9.60             | 7.80 | 8.46 | 4.20 | 6.47 | Ile177Thr (Homo) |
| 3      | BD      | 52 Y/F  | 48                | 20.68            | 8.15             | 6.15 | 8.02 | 3.25 | 6.12 | Ala178Thr/Arg49Trp |
| 4      | VJ      | 22 Y/F  | 38                | 20.94            | 7.64             | 6.54 | 7.40 | 2.34 | 5.45 | Ala178Thr (Homo) |
| 5      | UV      | 18 M/F  | 35                | 17.52            | 5.68             | 4.18 | 4.92 | 2.28 | 2.58 | Arg57Gln/Gly75 Ser |
| 6      | SH      | 18 Y/M  | 37                | 17.34            | 4.98             | 3.68 | 4.16 | 1.78 | 3.66 | Arg49Trp (Homo) |
| 7      | AB      | 14 Y/M  | 22                | 21.76            | 9.54             | 7.64 | 8.62 | 4.54 | 7.32 | Ala178Thr (Homo) |
| 8      | BK      | 33 Y/M  | 18                | 20.82            | 10.51            | 8.61 | 9.86 | 5.21 | 7.84 | Ala178Thr/Homo |
| 9      | DB      | 14 Y/F  | 3                 | 22.60            | 9.86             | 7.81 | 8.52 | 3.41 | 7.62 | Pro144Ser (Homo) |
| 10     | VB      | 45 Y/M  | 3                 | 19.72            | 8.34             | 6.94 | 7.98 | 2.74 | 6.58 | Val252Met (Homo) |
| 11     | SK2     | 29 D/M  | 33                | 18.44            | 7.56             | 6.26 | 7.04 | 4.06 | 4.84 | Ala178Thr/Arg49Trp |
| 12     | DR      | 49 Y/M  | 17                | 21.87            | 9.46             | 7.46 | 8.54 | 5.46 | 7.34 | Arg49Trp (Homo) |
| 13     | MM      | 38 Y/F  | 3                 | 23.74            | 10.89            | 7.23 | 9.76 | 3.43 | 8.46 | Met176Ile (Homo) |
| 14     | NM      | 6 Y/M   | 19                | 19.86            | 8.28             | 7.08 | 7.86 | 2.18 | 6.16 | Met176Val (Homo) |
| 15     | AT      | 10 Y/M  | 20                | 25.73            | 12.56            | 9.87 | 11.63 | 6.67 | 9.53 | Met176Val (Homo) |
| 16      | PM      | 21 Y/M  | 53                | 17.83            | 6.58             | 5.26 | 7.13 | 2.26 | 6.53 | Met176Val (Homo) |
| 17      | SK3     | 42 Y/M  | 52                | 21.73            | 9.76             | 7.76 | 8.34 | 2.46 | 7.37 | Arg49Trp (Homo) |
| 18      | BD      | 24 Y/M  | 40                | 18.42            | 7.62             | 6.02 | 7.28 | 1.32 | 6.18 | Arg49Trp (Homo) |
| 19      | MP      | 35 Y/M  | 45                | 18.74            | 6.48             | 5.38 | 7.12 | 2.88 | 6.19 | Arg159Pro (Homo) |

Methemoglobinemia Type-II

| SL. No. | Case ID | Age/sex | Meth-Hb level (%) | NADH-Cyb5r activity in fresh sample (IU/g Hb) | NADH-Cyb5r activity after 1 week (IU/g Hb) | NADH-Cyb5r activity after 2 week (IU/g Hb) | Genotypes |
|--------|---------|---------|-------------------|---------------------------------------------|-------------------------------------------|------------------------------------------|------------|
|        |         |         |                   | Spectrophotometer | 96-well plate method | 4°C | −20°C | 4°C | −20°C | |
| 20     | SS      | 5 Y/F   | 51                | 16.94            | 5.68             | 4.68 | 5.18 | 2.58 | 4.20 | Arg49Trp (Homo) |
| 21     | VS      | 8 Y/M   | 42                | 17.98            | 7.95             | 5.45 | 6.98 | 3.15 | 5.58 | Gly75Ser (Homo) |
| 22     | BP      | 25 D/M  | 32                | 16.34            | 4.52             | 3.62 | 4.05 | 1.32 | 3.52 | Gly75Ser (Homo) |
| 23     | SK      | 3 Y/F   | 52                | 20.52            | 7.50             | 5.20 | 7.13 | 2.52 | 6.13 | Trp233Ter (Homo) |
| 24     | AN      | 4 Y/F   | 15                | 21.85            | 8.60             | 5.32 | 7.54 | 3.60 | 6.16 | Trp233Ter (Homo) |
| 25     | SV      | 11 M/M  | 31                | 21.74            | 7.42             | 7.56 | 8.46 | 3.40 | 7.16 | Cys204Arg (Homo) |

Enzyme activity ranged (mean ± SD)

| Enzyme activity | Mean ± SD | Mean ± SD |
|-----------------|-----------|-----------|
| Spectrophotometer | 13.42–21.58 | 6.09–10.07 |
| 96-well plate method | 4.67–7.93 | 5.70–9.28 |

Enzyme activity of normal control ranged using microplate reader showed 13.42–21.58 IU/g Hb (mean ± SD: 17.5 ± 4.08 IU/g of Hb) and NADH-Cyb5r deficient cases ranged between 6.09 and 10.07 IU/g Hb (mean ± SD: 8.08 ± 1.99 IU/g Hb).
In the system in which the light path of the cuvette is 1 cm, the enzyme activity (E) in IU/g hemoglobin is:

\[ E = 100 \times A/\text{Hb} \]

where \( A \) is number of enzyme units per ml and \( \text{Hb} \), the concentration of hemoglobin in g/dl.

**Checking stability of the hemolysate**

**Storing hemolysate at 4 and −20°C**

A total of 25 normal samples were used for this purpose. All these hemolysate were stored at −20 and 4°C. The activity was checked after a time period of 1 week and 2 weeks. This study was done to check stability of the hemolysate stored at two different temperatures for a period of 2 weeks. Four aliquots were made for each sample. Two were stored at 4°C and the other two at −20°C. Activity was checked for every aliquot after a time period of 1 week and 2 weeks. For all the above parameters, the total volume of reaction mixture was taken as 200 µl. All the components of the reaction mixture were added in the proportion described before for microplate reader and this was same for all the samples.

**Analysis at variable volume of reaction mixture**

A total of 25 samples were used for this study. The purpose of this study was to check suitability of minimum total volume of the reaction mixture. Comparison was made using same samples. Analysis at variable volume of reaction mixture was done using 50, 100, and 200 µl.

**Data analysis**

The data were analyzed qualitatively and quantitatively. Qualitatively is by seeing the graph of optical density change per minute on screen of microplate reader of each sample. Quantitatively, change in optical density is measured 340 nm for 10 minutes through the absorbance and used for calculation of enzyme activity. Line graph of change in optical density was created using the software MAGELLAN V6.5 in the TECAN Infinite 200 PRO microplate reader.

**Statistical analysis**

After the calculation of arithmetic means and SD of the mean and t-test were administered for comparison of NADH-CYB5R activity between spectrophotometer measurement and 96-well plate method using SPSS version 20 software (SPSS, Inc., Chicago, IL, U.S.A). \( p < 0.05 \) was significant.

**Results**

Enzyme activity in all the 25 NADH-CYB5R deficient samples ranged from 6.09 to 10.07 IU/g Hb (mean ± SD: 8.08 ± 1.99 IU/g Hb) and blood bank normal control samples had a normal range between 13.42 and 21.58 IU/g Hb (mean ± SD: 17.5 ± 4.08 IU/g of Hb). The use of a microplate reader minimizes sample processing time (~25 seconds per sample), reagent consumption (~178 µl per sample), and sample volume (~22 µl). Data obtained from the plate reader were compared to those obtained, under similar conditions, in a conventional spectrophotometer. It was observed that the normal range for enzyme activity is different for the two different instruments. Normal range by using standard UV/visible spectrophotometric method was found between 30.05 and 40.49 IU/g Hb (mean ± SD: 35.27 ± 5.22 IU/g of Hb) where as Tecan’s microplate reader showed 13.42–21.58 IU/g Hb (mean ± SD: 17.5 ± 4.08 IU/g of Hb). Statistical analysis revealed that there were no significant differences between the two methods (\( p \) value > 0.05). Results showed that the microplate method allow to differentiate between normal individuals, homozygous patients, and individuals having an intermediate enzyme activity. During screening, 25 samples showed significantly decreased activity by microplate method using 200 µl reactions. The difference between enzyme activities which is normal, intermediate and deficient can be observed from the graphs itself (Fig. 1(a–c)). The normal sample was shown a steady as well as sharp diagonal line of absorbance, whereas, the sample with intermediate activity was shown somewhat less sharp decrease, while the deficient sample was shown very little decrease or straight line of activity. A large difference in the activity of the normal and deficient sample can be easily seen from the graph. In fact, it can be inferred from the graph itself whether the subject is deficient or normal or having an intermediate level of activity (Fig. 1). Hemolysate was stored at 4 and −20°C and activity was checked after duration of 1 week and 2 weeks. The results obtained are shown in Table 1, samples showed no significant decrease in the enzyme activity even after storing for 1 week at −20°C, there was 7.30% decrease in the enzyme activity whereas samples store at 4°C significantly lost their activity (22.02%). Enzyme activities of fresh samples and activities of the same samples, after storing for 1 week and 2 weeks, at 4 and −20°C are shown in bar diagram (Fig. 1(d)). It is observed from the Table 1, shown that samples had significant loss of activity when stored at 4°C and retained considerable activity when stored at −20°C for 1 week. Reaction volume of 100 and 50 µl did not give any consistent or repeatable results (data not shown).
The graphs obtained showed an absurd pattern. They were not as clear and sharp as obtained with total reaction volume of 200 μl whereas total volume of 200 μl gave optimum results.

**Discussion**

NADH-CYB5R, present in various tissues of the body, is a redox enzyme of multiple functions. The deficiency of the enzyme leads to hereditary methemoglobinemia. Two types of RCM are observed. In type I RCM, the enzymatic defect is limited to the erythrocytes. Methemoglobinemia can be diagnosed by co-oximetry, methemoglobin concentration, NADH-CYB5R activity determination [12] and checking for absorption spectrum of methemoglobin for presence of Hb-M. In addition, the presence of Hb-M can be established by electrophoresis at pH 7.1, differential ultra violet spectrometry and DNA sequencing [13]. A few other tests to detect presence of methemoglobinemia have been described: In a simple spot test [5], whole blood is added to a mixture containing a hemolyzing agent, NADH, and indicator dye DCIP. In the presence of NADH diaphorase enzyme, the dye is reduced by NADH. During the reaction, NADH, which fluoresces when illuminated by long wavelength UV light, is oxidized into NAD, which is not fluorescent under such conditions. Depending on the time required to reduce the dye, the enzyme level can be determined qualitatively. This method is very easy to perform, less time is required, minimum requirements for the experiments, but quantitative measurement of the enzyme activity is not obtained. In an antibody based spot test [8], monoclonal antibodies to CYB5R was dot-blotted on nitrocellulose (NC) squares or strips, 1 μl per square. They were used to capture and enrich CYB5R from hemolysate, and the captured NADH-CYB5R activity was subsequently visualized with the substrate 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide. Serial dilutions of the hemolysate were made and used for the test. On the basis of intensity of the color developed after the reaction, and the least dilution showing the colored reaction, levels of the enzyme present in an individual could be determined. This method is also easy to perform; time required is less, sensitive and reliable. However, quantitative measurement of the enzyme activity is not obtained in this method. In a double-antibody sandwich ELISA [14], a polyclonal anti-CYB5R antibody was used for coating the plate and enzyme-labeled anti-CYB5R monoclonal antibody as a reporter. A standard graph was obtained by using standard CYB5R antigens. The concentration of NADH-CYB5R in an unknown sample (normal, heterozygote, or a patient) can then be determined from the standardized graph. The intensity of the color developed due to enzyme-substrate reaction is proportional to concentration in an unknown sample, which can be established from the standardized graph. This method, being very specific, highly sensitive and reliable, and able to make quantitative measurement of enzyme concentration, is advantageous over above two methods. However, cost involved in the production of the monoclonal antibodies is a major drawback of this method.
It has been established that NADH-CYB5R activity, both in soluble form and membrane bound form, in the erythrocytes of RCM patients is greatly reduced [15]. Heterozygote relatives of such patients also have lowered red cell NADH-CYB5R activity, approximately half the level of normal subjects [16]. To test the utility of our new method, it was applied to the semi quantitative detection of NADH-CYB5R activity in hemolysate from different subjects including one normal adult, one neonate, one type I RCM patient and heterozygous parents. Comparing the maximal detectable dilution of all hemolysate, it was found that the hemolysate NADH-CYB5R activity levels of both the neonate and their parents were much lower than that of the normal adult, and NADH-CYB5R activity in the patient’s hemolysate was the lowest of all the subjects. This microplate result coincided with the result obtained by a standard quantitative spectrophotometric method, which measures the diaphorase activity of NADH-CYB5R (Table 1). More convincingly, both results agreed with the generally accepted view on the NADH-CYB5R levels of normal adults, newborns, RCM patients and heterozygous relatives of such patients.

In conclusion, the new microplate techniques offer an alternative to standard method. The use of a microplate reader minimizes sample processing time, reagent consumption and sample volume ultimately decreases the cost and time of processing while maintaining high precision and sensitivity. Our method would be useful for the laboratory diagnosis of congenital methemoglobinemia. It is readily applicable to quantitative assay on pediatric sample as well as large number of samples for population screening.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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Notes on contributors

Prabhakar Kedar planned for experiment and design of the project and wrote the manuscript.

Anand Desai performed the lab work and experiments.

Prashant Warang performed lab experiments and data analysis.

Roshan Colah supervised and checked the final draft of manuscript.

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