# Minimize error in the evaluation of enzymatic activity

Marcomaria Zora[a,*], Emanuela Maria Marsana[a,*], Elisa Messina[a], Daniele Francofonte[a]

[a]*Institute for Biomedical Research and Innovation (IRIB-CNR), Via Ugo La Malfa, 153, Palermo, 90146, , Italy*
** these authors contributed equally.*

## 1. Introduction

Enzyme assays are an extremely useful investigative instrument in patients with clinical suspicion of lysosomal storage disease (LSD) to measure functional enzyme deficiency; combined with genetic analysis, they allow to get a more complete diagnostic picture [1][2]. Advances in the treatment of LSDs and the consequent interest in speeding up the diagnostic investigation make it necessary to find a rapid and effective method to identify affected patients, shortening the time to diagnosis and then improving the course of the disease, thanks to available therapies [3].

Measurement of enzyme activity using fluorometric techniques and DBS on filter paper is considered a reliable and cost-effective method [4][5].
The use of DBS has provided many advantages, including reduced blood manipulation and the consequent reduced biological risk, making it the reference method for the diagnosis of LSD [6]. Moreover, enzyme activity on DBS are stable, if correctly stored, a substantial advantage allowing samples to be transported safely over long distances [7][8]. For many years sugar derivatives of 4-methylumbelliferone (4MU) have been favorite substrates for the measurement of lysosomal enzyme activities, in a wide variety of cell and tissue [2]. Hydrolysis of these artificial substrates at acidic pH leads to the formation of 4-methylumbelliferone, which is strongly fluorescent ($\lambda_{ex}$ 365 nm, $\lambda_{em}$ 448 nm) at pH above 10 [9]. To date, these assays are performed using the protocol developed by Chamoles, which uses fluorescent substrates conjugated to 4MU and a single calibration curve with increasing concentrations of the fluorochrome [10][11][5]. However, the results are affected by fluorescence quenching by hemoglobin, which, together with the small sample size, can result in a low light emission signal. In addition, the sample's own characteristics such as type of hemoglobin, storage, and mode of preparation, affect the value of enzyme activity and may lead to a faked value when a single standard calibration curve is used. The most accurate method is to prepare one calibration curve for each sample, using the corresponding DBS but this requires more DBSs for a sample and reduces the usable space on the multi-well plate.

In this report we show a new, quick and easy-to-apply calibration method that accounts for sample variability while providing an accurate measure of enzyme activity, optimizing available space and reducing analysis time.

The method was tested and applied to the quantization of $\alpha$-galactosidase A, whose enzyme deficiency causes Fabry disease [12].
Due to lyonization process, female Fabry patients usually manifest a variable symptomatology, ranging from asymptomatic to severe phenotypes, and GLA activity can be in the normal range, even in the presence of causative mutations; for this reason the GLA assay is reliable only in male patients [13].

*Corresponding author
*Email address:* marcomaria.zora@cnr.it (Marcomaria Zora)

## 2. Materials and methods

### *2.1. Samples*

From a previous analysis, we selected DBS of 12 male subjects from all over Italy, with different values of $\alpha$-GalA enzyme activity and different quenching coefficients so as to better highlight the differences in enzyme activity obtained by the three calculation methods described below. For all samples, GLA gene sequencing was performed. Samples S1, S4, S8 and S11 are affected by Fabry disease and carry the causative mutations R112H, R301G, R301Q and R356W respectively, the other eight samples are healthy. Hemizygotes affected with FD had $\alpha$-GalA activities below 1.7 $\mu mol/h/L$ [4]. Blood samples are placed on filter paper and left to dry 3-4 hours at room temperature. DBS are then stored at 4°C before analysis so as to preserve the integrity of the enzyme [14]. DBS are stored and transported with special care, because humidity and high temperatures can affect results [7].

### *2.2. Reagents and solutions preparation*

- Citrate-phosphate buffer pH 4.5, CPB (0.1M citric acid, 0.2M dibasic sodium phosphate).
- 4-methylumbeliferyl $\alpha$-D-galactopyranoside, synthetic substrate (Sigma Aldrich)
- N acetyl-D-galactosamine, NAG, Inhibitor of enzyme Isoform B (Sigma Aldrich). NAG is used to eliminate the contribution of the enzyme isoform of our interest, which, being compatible with the synthetic substrate used, would interfere with the evaluation of the activity [15] [16]
- 4-Methylumbelliferone, 4MU, Fluorescent reagent (Sigma Aldric)
- Ethylenediamine 99%, EDM (Sigma Aldric)
- Solution S: prepared using substrate 7.28 mmol/L in CPB
- Solution A, for active wells: prepared with substrate 3.64 mmol/L and NAG 92.8 mmol/L in CPB
- Solution B, for background wells: prepared with NAG at concentration 92.8 mmol/L in CPB
- Stop Solution: prepared with EDM 0.1M in $H_2O$ milliQ
- Calibration Solution, CAL: prepared with 4MU 50 pmol/µl in Stop Solution
- Calibration Curve Solutions, $C_0$, $C_1$, $C_2$, $C_3$: prepared with increasing concentrations of 4MU, 0, 2.5, 5, 10 pmol/µl in Stop Solution.

### *2.3. Enzyme assays*

For each sample, eight DBS are arranged in a column, in a black 96-well flat-bottom plate, positioned according to the scheme shown in Figure 1.
In each column there are two active wells (A green) in which the enzyme, if functioning, catalyzes the substrate and two background wells (B red) in which the enzyme has no catalytic activity because the substrate is added only at the end of the reaction, just before the fluorometer reading, as suggested in the literature [17]). The last 4 wells of column will be used for the sample-specific calibration curve. Then, 100 µl of Solution A are dispensed in the active wells and 100 µl of Solution B in the background wells and in the calibration curve wells. To evaluate self-degradation of the substrate, the solution S was incubated separately and used at the end of the reaction. The plate and the solution S was incubated in a Thermo-Mixer at

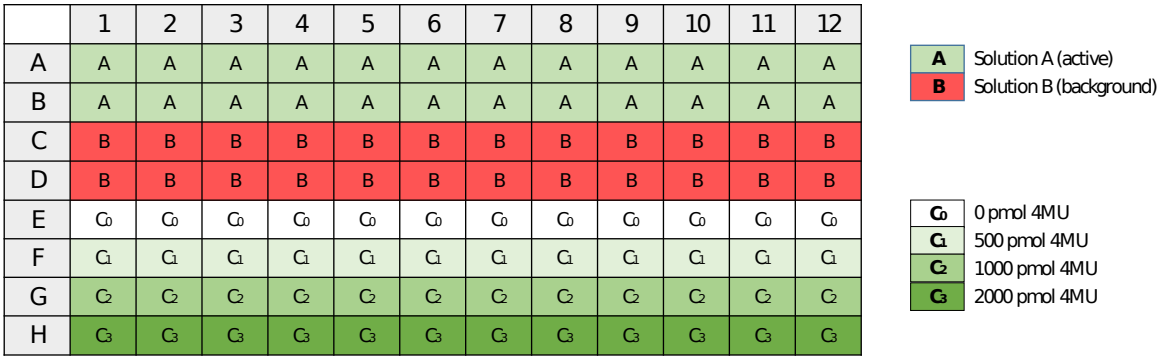


Figure 1: Layout of samples in the 96-well plate. The wells with the letter A are filled with solution A, and in these wells the enzyme is active. The wells with the letter B are used to evaluate the background. In these wells the enzyme is not active because there is no substrate during incubation. Wells $C_0, C_1, C_2, C_3$ are filled with solution B and dedicated to calibration curves.

37°C, 900 rpm for 18h, with aluminum foil to reduce evaporation. At the end of incubation, 50 µl of Solution S was added to the background wells and calibration curves wells; 50 µl of CPB are then added to active wells to balance the volumes. Finally, 200 µl of Stop Solution wad added only to the active and background wells, to stop the enzymatic reaction [18] and 200 µl of Calibration Curve solutions $C_0$, $C_1$, $C_2$, $C_3$ were dispensed as shown in Figure 1.
The plate was finally mixed at 25°C, 900 rpm, for an additional 10 minutes. The low temperature and especially the high pH, block the catalytic activity and amplify the fluorescence signal [19]. The BMG LABTECH's Clario Star Plus fluorometer is used to scan the plate by setting the specific excitation and emission wavelength of the 4MU ($\lambda_{ex}$ 365 nm, $\lambda_{em}$ 448 nm) [9].

### *2.4. α-GalA activity*

Enzyme activity $A$, is defined as $Q$ picomoles of substrate transformed (or product formed) per hour per microliter of blood [8].

$$A = \frac{\Delta Q}{\Delta t} \cdot \frac{1}{\mu} \simeq \frac{Q}{t \cdot \mu} \tag{1}$$

The relationship between the quantity $Q$ and fluorescence $F$ is linear

$$F = \alpha \cdot Q + F_b \tag{2}$$

$$F - F_b = \alpha \cdot Q$$

where $\alpha$ is the coefficient that indicates how much the fluorescence increase when the amount of fluorophore in the sample increase; it also depends on hemoglobin quenching as well as on the nature of reagents themselves. $F_b$ is the background fluorescence which does not depend on enzymatic activity but, for example, to substrate self-degradation and intrinsic fluorescence of solutions. This value coincides with the fluorescence in the wells where the enzyme not work. The relation 2 is the so-called calibration curve, that allows to derive from the fluorescence values, the amount of 4MU molecules produced during the reaction. From the 2, indicating with $F_a$ the fluorescence of the active wells at the end of the reaction, we obtain

$$Q = \frac{F_a - F_b}{\alpha} \tag{3}$$

The enzyme activity is then

$$A = \frac{F_a - F_b}{\alpha \cdot t \cdot \mu} \tag{4}$$

where $t$ is the time taken for the reaction and $\mu$ the total amount of blood in the well that actually participates in the reaction; this parameter was derived empirically as described in appendix. The enzyme activity is calculated using three different methods for evaluation of $\alpha$ coefficients. The first method consists of preparing a calibration curve for each sample and using the angular coefficient $\alpha$, obtained from the best fit of the points. With the second method, the calculation was performed using for all samples the $\alpha$ coefficient obtained from a randomly chosen curve. The third method is the **fast calibration**, object of this paper, which does not require a true calibration curve but allows us to obtain a different $\alpha$ coefficient for each sample as described below.

### *2.5. Fast Calibration method*

Fast calibration consists of a different method used to estimate the $\alpha$ coefficient. This, from the relation 2, is given by

$$\alpha = \frac{\Delta F}{\Delta Q}$$

Then, by adding a known amount $\Delta Q$ of fluorescent molecules in a given well and measuring the $\Delta F$ change in fluorescence, we can derive $\alpha$. This operation is performed, after the first fluorometric scan, on the background wells by adding 10 $\mu$l of CAL solution (a volume of liquid that is negligible compared to the total) containing precisely 500 pmol of 4MU.
A second scan is then performed after shaking the plate for 5 min at 900 rpm at 25°C. To better highlight the fluorescence change (maximizing the signal-to-noise ratio), it is convenient to perform this operation in the Background wells only, where the fluorescence $F_b$ is lower.
Denoting by $F_b^*$ the fluorescence of the Background wells after adding 500 pmol of 4MU, we obtain

$$\alpha = \frac{F_b^* - F_b}{500} \tag{5}$$

At the numerator we have the fluorescence "change" and at the denominator the total "change" of 4MU molecules in the well. In this way, we are able to derive the correct $\alpha$ coefficient for each sample without the need to have a calibration curve for each one. By putting together the 4 with the 5 we can also write

$$A = \frac{F_a - F_b}{F_b^* - F_b} \cdot \frac{500}{t \cdot \mu} \tag{6}$$

Where $500/(t \cdot \mu)$ is a constant for all samples.

## 3. Results

We selected twelve subjects, with different values of $\alpha$-GalA activity and different quenching coefficients ($\alpha$ coefficients), to better highlight the differences in the enzymatic activity obtained from the three methods described. As can be seen in Fig.2, the $\alpha$ coefficients (the slopes of the different calibration curves) differ significantly from sample to sample.

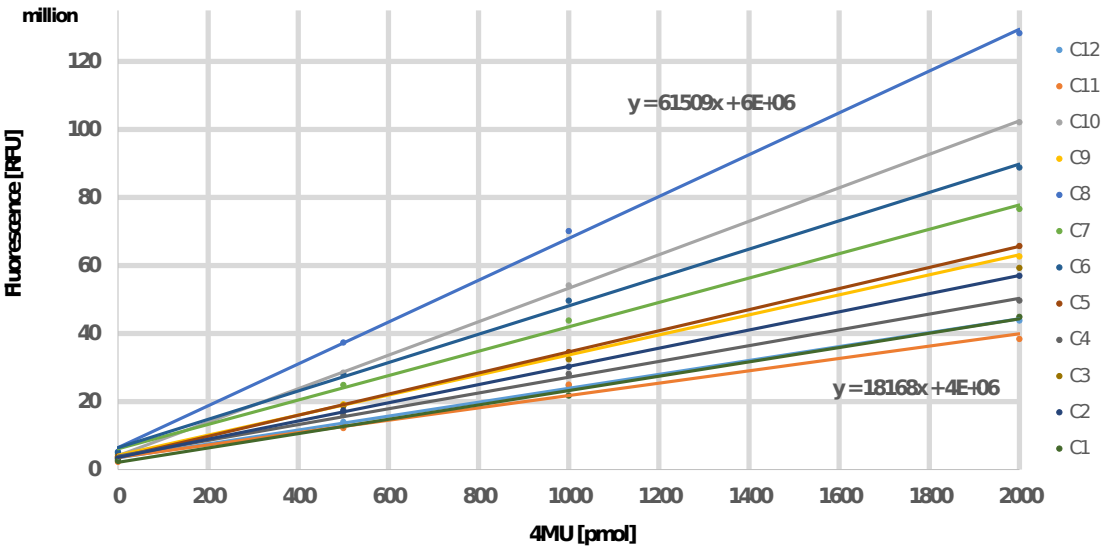


Figure 2: The x-axis shows the total amount of fluorescent 4MU molecules in the calibration wells and the y-axis shows the corresponding fluorescence. The colored lines represent the calibration curves for each sample. The angular coefficient, resulting from linear regression, is the $\alpha$ coefficient used to calculate enzyme activities with the first method. The slopes of the different calibration curves differ significantly from sample to sample.

With the first method, activities were calculated using for each sample its own calibration curve. These values are reliable and were used as a reference to compare the other two methods, we call these values "true values". With the second method (the most used method and generally considered the standard one), the calculation was done using a single calibration curve, randomly chosen from all samples. To assess how this might affect the enzymatic activity, the calculations were repeated using all 12 curves. The results are shown in Table 1.

Table 1: Enzyme activity as a function of the 12 calibration curves. Samples are indicated with S1, S2, etc... and calibration curves with C1,C2, etc.... On the diagonal are highlighted, with a circle, the activities of each sample calculated using its own calibration curve.

| Samples | Calibration Curves | | | | | | | | | | | | Err. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | C1 | C2 | C3 | C4 | C5 | C6 | C7 | C8 | C9 | C10 | C11 | C12 | δ |
| S1 | 0,2 | 0,2 | 0,2 | 0,2 | 0,2 | 0,1 | 0,1 | 0,1 | 0,2 | 0,1 | 0,3 | 0,2 | 0,1 |
| S2 | 22,2 | 17,5 | 16,8 | 20,1 | 15,1 | 11,2 | 13,0 | 7,6 | 15,9 | 9,5 | 25,7 | 22,8 | 5,4 |
| S3 | 29,7 | 23,4 | 22,6 | 27,0 | 20,2 | 15,0 | 17,5 | 10,2 | 21,2 | 12,7 | 34,5 | 30,6 | 7,2 |
| S4 | 0,7 | 0,5 | 0,5 | 0,6 | 0,5 | 0,3 | 0,4 | 0,2 | 0,5 | 0,3 | 0,8 | 0,7 | 0,2 |
| S5 | 39,2 | 30,9 | 29,8 | 35,6 | 26,6 | 19,8 | 23,0 | 13,4 | 28,0 | 16,8 | 45,4 | 40,4 | 9,8 |
| S6 | 31,7 | 25,0 | 24,1 | 28,8 | 21,5 | 16,0 | 18,6 | 10,9 | 22,7 | 13,6 | 36,8 | 32,7 | 10,7 |
| S7 | 20,5 | 16,2 | 15,6 | 18,7 | 14,0 | 10,4 | 12,1 | 7,0 | 14,7 | 8,8 | 23,8 | 21,2 | 5,9 |
| S8 | 1,0 | 0,8 | 0,8 | 0,9 | 0,7 | 0,5 | 0,6 | 0,4 | 0,7 | 0,4 | 1,2 | 1,1 | 0,5 |
| S9 | 16,5 | 13,0 | 12,5 | 15,0 | 11,2 | 8,3 | 9,7 | 5,7 | 11,8 | 7,1 | 19,1 | 17,0 | 4,0 |
| S10 | 29,8 | 23,5 | 22,6 | 27,1 | 20,2 | 15,1 | 17,5 | 10,2 | 21,3 | 12,8 | 34,6 | 30,7 | 11,8 |
| S11 | 0,3 | 0,3 | 0,3 | 0,3 | 0,2 | 0,2 | 0,2 | 0,1 | 0,2 | 0,1 | 0,4 | 0,4 | 0,2 |
| S12 | 13,5 | 10,6 | 10,2 | 12,2 | 9,2 | 6,8 | 7,9 | 4,6 | 9,6 | 5,8 | 15,6 | 13,9 | 5,1 |

For each sample $S_i$ we get 12 different activity values (reported in the rows of the Table 1), one for each calibration curve $C_i$. In the diagonal are the true values, calculated for each sample using its own calibration curve, highlighted with a circle. The last column shows the standard deviation from the true value for each sample. These numbers represent the error associated with the second method.
To better underline the results of Table 1, we have plotted the activity values in the graph in Figure 3. It is evident how these values change considerably when different calibration curves

are chosen. This shows the importance of choosing the right calibration curve if you want to reduce the error. As can be seen from the graph, in our sample set, in each case the separation between sick and healthy is clear. However, reducing the error near the cut-off may be helpful in reducing the number of false positives.

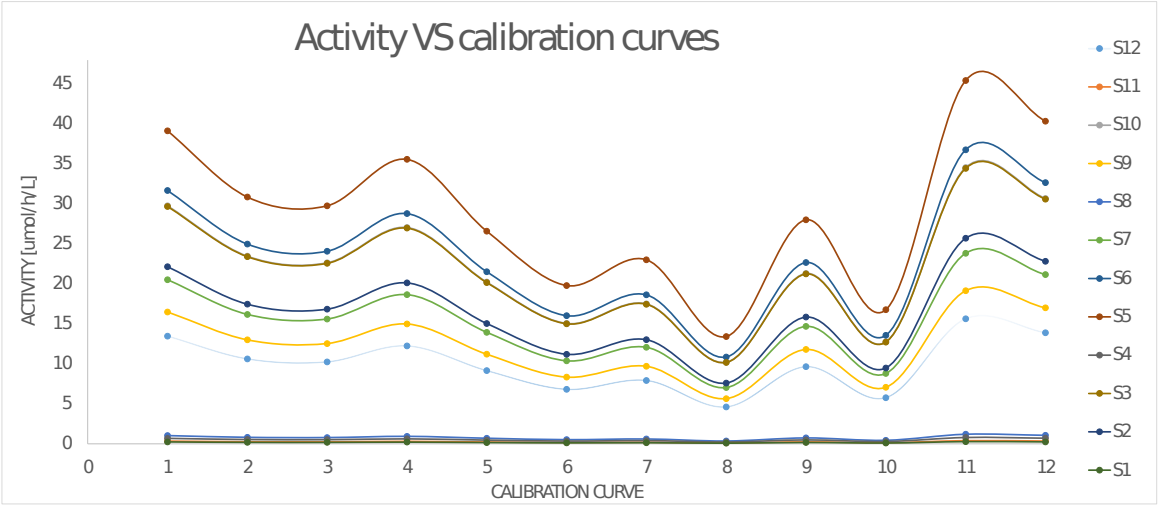


Figure 3: Activity values for each sample as a function of the chosen calibration curve. The lowest activity values are those referring to Fabry patients.

Table 2 shows a comparison of enzyme activity values obtained with the three different methods; for the second method minimum and maximum represent the lowest and highest values of activity obtained with the different curves, respectively. The error associated with the second method is the standard deviation from the true value. The values obtained by the third method are very close to the true values obtained by the first method, and the errors associated with that values is of the same order of magnitude as that of the first method.

Table 2: Comparison of enzyme activity calculated with the three different methods. The columns labeled Minimum and Maximum show the minimum and maximum activity values, obtained with the different calibration curves used. Patients with Fabry disease are highlighted in the Samples column. The column corresponding to the third Method shows errors close to those obtained by the first method.

| Samples | METHOD 1 TRUE VALUES | | METHOD 2 THE MOST USED | | | METHOD 3 FAST CALIBRATION | |
|---|---|---|---|---|---|---|---|
| | Activity [µmol/l/h] | Error | Activity [µmol/l/h] Minimum | Activity [µmol/l/h] Maximum | Error | Activity [µmol/l/h] | Error |
| S1 | 0,2 | 0,1 | 0,1 | 0,3 | 0,1 | 0,2 | 0,1 |
| S2 | 17,5 | 2,0 | 7,6 | 25,7 | 5,4 | 16,6 | 2,3 |
| S3 | 22,6 | 2,0 | 10,2 | 34,5 | 7,2 | 22,0 | 2,1 |
| S4 | 0,6 | 0,2 | 0,2 | 0,8 | 0,2 | 0,6 | 0,2 |
| S5 | 26,6 | 1,6 | 13,4 | 45,4 | 9,8 | 26,4 | 2,6 |
| S6 | 16,0 | 2,5 | 10,9 | 36,8 | 10,7 | 16,0 | 3,8 |
| S7 | 12,1 | 0,5 | 7,0 | 23,8 | 5,9 | 11,5 | 0,8 |
| S8 | 0,4 | 0,2 | 0,4 | 1,2 | 0,5 | 0,4 | 0,2 |
| S9 | 11,8 | 1,7 | 5,7 | 19,1 | 4,0 | 11,1 | 1,9 |
| S10 | 12,8 | 1,7 | 10,2 | 34,6 | 11,8 | 12,8 | 2,3 |
| S11 | 0,4 | 0,4 | 0,1 | 0,4 | 0,2 | 0,3 | 0,3 |
| S12 | 13,9 | 0,6 | 4,6 | 15,6 | 5,1 | 14,0 | 1,1 |

In Table 3 the alpha values, calculated both with the best fit of calibration curves and with the Fast Calibration method, are shown. The values obtained are very similar.

Table 3: Comparison between $\alpha$-values derived by best fit of the calibration curves and those obtained by the fast calibration method.

| | S1 | S2 | S3 | S4 | S5 | S6 | S7 | S8 | S9 | S10 | S11 | S12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| α - True values | 21087 | 26741 | 27731 | 23201 | 31035 | 41693 | 35853 | 61509 | 29463 | 49221 | 18168 | 20454 |
| α - Fast calibration | 21712 | 28136 | 28430 | 25189 | 31267 | 41863 | 37656 | 62140 | 31321 | 49239 | 22614 | 20334 |

## 4. Discussion

In the diagnosis of lysosomal storage diseases, it is important to assess the activity of the enzyme that is presumed to be the cause. A simple, fast and accurate method is needed to process a large number of samples. The technique commonly used in this type of analysis is fluorometric analysis, using dried blood samples (DBS) [10]. Fluorescence, however, is affected by the characteristics of the blood, so it is correct to prepare a calibration curve for each patient, but this extends the processing time and reduces the available space on the plate. The most widely used method, the second method described in this paper, involves a single calibration curve for all samples, and this leads to lower precision of results. In fact it can be seen how the choice of calibration curve affects the activity values, that show great variability and deviate more or less markedly from the true values. With the Fast Calibration, we achieve high accuracy without the need to prepare any calibration curve and without resorting to hemoglobin precipitation. Results demonstrates that this method is valid and that it is possible to obtain measurements with high precision easily, quickly and by maximizing the useful space in the plate. With a 96-well plate, for example, 24 different samples in duplicate can thus be processed.

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