Supplementary Material

Development of Stabilizing Hemoglobin A2 Reference Materials and Assessment of Commutability using Multiple Statistical Models

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Supplementary Material 1

1. Evaluation protocol

1.1 Precision study

Intra- and inter-assay precision were evaluated in accordance with the CLSI EP5-A2 document(1). For the evaluation of intra-assay precision, the same lot of Eluent kit was used for 20 quality control tests on the calibrated analyzer. For the inter-assay precision evaluation, quality control was measured 20 times via three lots of the eluent kit on a calibrated analyzer. The mean, CV and SD were then calculated.

1.2 Accuracy study

Accuracy was evaluated in accordance with the CLSI EP10-A3 guidelines(2). Human primary HbA2 reference materials (ERM-DA485 and ERM-DA486) were measured five times on a calibrated analyzer. The mean, standard deviation (SD) and relative deviation (RD) were then calculated.

1.3 Linearity study

The linearity assessment was performed in accordance with the CLSI EP6-A guidelines(3). Two blood specimens were included: one with a low HbA2 value (HbA2: 2.10%) and one with a high HbA2 value (HbA2: 7.30%). The samples were then diluted 120-fold and mixed at ratios of 2:0, 1.6:0.4, 1.2:0.8, 1:1, 0.8:1.2, 0.4:1.6 and 0:2, creating a total of seven concentrations with low- and high-HbA2 blood pools. Seven mixed samples were then obtained and measured three times on the calibrated analyzer.

1.4 Carryover study

Carryover was evaluated in accordance with the CLSI EP10-A3 guidelines(2). Briefly, blood samples with low and high HbA2 values (2.10% and 6.60%, respectively) were measured as follows. Low- and high-value blood samples were injected repeatedly (L1–L5 and H1–H4) and subsequently measured in the order L1–L2–L3–L4–H1–H2–H3–H4–L5. The data were analyzed to determine carryover. All results are available in Table S3.

Supplementary Material 2

2. Associated uncertainty assessment

The sources of uncertainty were determined in accordance with ISO Guide 35:2024(4). This included certified values (i.e., measurement precision, bias, and internal standard control materials), as well as homogeneity and stability (i.e., long-term, shipping, opening the bottle, freeze–thaw cycles, cold storage, and room temperature storage). The associated uncertainty analysis was performed using one hundred randomly selected vials of HbA2 reference materials, which were stored at -70°C.

2.1 Uncertainties from assignment

2.1.1 Evaluation of measurement precision uncertainty

The HbA2 reference materials were tested in ten vials per day, three times per bottle, over five days, using the above measurement system at temperatures between 18 and 25°C and humidity levels between 15 and 75%. The measurement precision uncertainty data were calculated via the following formula:

$$\bar{x} = \frac{\sum_{i=1}^{n} x_i}{n}$$

$$u_{rep.rel} = \frac{1}{\bar{x}} \sqrt{\frac{1}{n(n-1)} \sum_{i=1}^{n} (x_i - \bar{x})^2}$$

where $\bar{x}$ is the total mean; x_i is the measured value; n is the testing number; and $u_{rep.rel}$ is the precision uncertainty.

2.1.2 Evaluation of working calibrator uncertainty

The combined uncertainties of the working calibrators (human HbA2 reference materials (ERM-DA485 and ERM-DA486)) were calculated via the following formula:

$$u_{wcal} = \sqrt{\frac{u_{c1}^2 + u_{c2}^2}{2}}$$

$$u_{wcal.rel} = \frac{u_{wcal}}{k \times C_{wcal}}$$

where u_{c1} is the expanded uncertainty of the low-value calibrator; u_{c2} is the expanded uncertainty of the high-value calibrator; u_{wcal} is the combined expanded uncertainty; C_{wcal} is the calibrator determination; $u_{wcal.rel}$ is the standard uncertainty; and k is the inclusion factor (k=2).

2.1.3 Uncertainties of Assignment

The uncertainty of the certified values of the control materials was analyzed with the following formula:

$$u_{char.rel} = \sqrt{u_{rep.rel}^2 + u_{wcal.rel}^2}$$

$$u_{char} = u_{char.rel} \times C$$

where $u_{char.rel}$ is the relative uncertainty of all the certified factors; u_{char} is the standard uncertainty of all the certified factors; and C represents the initial certified values.

2.2 Uncertainty of homogeneity

Fifteen vials of HbA2 reference materials were used to assess homogeneity via stratified random sampling from the total sample, which was stored at -70°C. The sequence of between- and within-bottle variances measured was as follows: 1, 3, 5, 7, 9, 11, 13, 15, 2, 4, 6, 8, 10, 12, 14, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, 1. The measured data were analyzed via one-way ANOVA via SPSS 16.0, and the uncertainty of homogeneity was calculated via the following formula:

$$u_{bb} = \frac{1}{\bar{x}} \sqrt{\frac{(MS_{bb} - MS_{wb})}{n}}$$

where $\bar{x}$ is the total mean; n is the replicate testing number; MS_{bb} is the mean square between bottles; MS_{wb} is the mean square in the bottle; and u_{bb} is the uncertainty of homogeneity.

2.3 Uncertainties from stability

2.3.1 Bottle opening stability

Ten vials containing HbA2 reference materials were selected from all the samples (stored at -70°C) via stratified random sampling to evaluate bottle opening stability. Two vials/level HbA2 reference materials were opened on days 1, 2, 3, 4, and 5. The samples were subsequently stored at 2–8°C. All the HbA2 reference materials were tested 3 times/vial on the 5th day. The uncertainty of bottle opening stability is u_{st1}.

2.3.2 Freeze–thaw stability

Ten vials/level HbA2 reference materials were used to assess freeze–thaw stability via stratified random sampling from total samples (stored at -70°C). Two vials/level HbA2 reference materials were subjected to 5 freeze–thaw cycles between -40°C and 2°C~8°C on the 1st, 2nd, 3rd, 4th, and 5th

days, respectively, and then stored at 2°C to 8°C. All the HbA2 reference materials were tested 3 times/vial on the 5th day. The uncertainty of the freeze–thaw stability is $u_{s\,t2}$.

2.3.3 Long-term stability

Thirty-two vials/level HbA2 reference materials were selected from all samples (stored at -70°C) via stratified random sampling to evaluate long-term stability. The samples were stored at -70°C. Two vials/level HbA2 reference materials were tested 3 times/vial at the 1st, 2nd, 3rd, 6th, 9th, 12th, 18th and 24th months. The uncertainty of long-term stability is $u_{s\,t3}$

2.3.4 Cold storage stability

Ten vials/level HbA2 reference materials were used to assess cold storage stability via stratified random sampling of total samples. Two vials/level HbA2 reference materials were selected on the 1st, 2nd, 3rd, 4th, and 5th days and then stored at 2°C–8°C. All the HbA2 reference materials were tested 3 times/vial on the 5th day. The uncertainty of the cold-storage stability is $u_{s\,t4}$

2.3.5 Shipped stability

Ten vials/level HbA2 reference materials, which were placed on a shaker to simulate transportation at -20°C, were selected from all the samples (stored at -70°C) via stratified random sampling to study shipped stability. Two vials/level HbA2 reference materials were tested 3 times/vial on the 1st, 2nd, 3rd, 4th, and 5th days. The uncertainty of shipped stability is $u_{s\,t5}$

2.3.6 Room temperature stability

Ten vials/level HbA2 reference materials were selected from all samples (stored at -70°C) by stratified random sampling, and were stored at 18°C to 25°C, which were used to evaluate the room temperature stability. Two vials/level HbA2 reference materials were tested 3 times/vial at the 1st, 2nd, 3rd, 6th, 12th and 24th hours. The uncertainty of the room temperature stability is $u_{s\,t6}$

2.3.7 Statistical analysis

The uncertainty of each stability was calculated via the following formula:

$$u_{s\,t} = t \times s\,(b_1)$$

$$b_1 = \frac{\sum_{i=1}^{n} (x_i - \bar{x})\,(\bar{y} - \bar{\bar{y}})}{\sum_{i=1}^{n} (x_i - \bar{x})^2}$$

$$b_0 = \bar{\bar{y}} - b_1 \bar{x}$$

$$s = \sqrt{\frac{\sum_{i=1}^{n} ((\bar{y} - b_0 - b_1 x_i)^2}{n-2}}$$

$$s(b_1) = \frac{s}{\sqrt{\sum_{i=1}^{n} (x_i - \bar{x})^2}}$$

where t is the total number of time points; $s(b_1)$ is the uncertainty of the slope; b_1 is the slope; b_0 is the intercept; s is the SD of the straight line; x_i is the number of time points; $\bar{x}$ is the mean of the time points; $\bar{y}_i$ is the mean of the time points; $\bar{\bar{y}}_i$ is the total mean of the time; and u_{stt} is the uncertainty of each stability.

2.4 Combined standard uncertainty

The standard uncertainty of the HbA2 reference materials was calculated via the following formula:

$$u_c = \sqrt{u_{chart}^2 + u_{bb}^2 + u_{st1}^2 + u_{st2}^2 + u_{st3}^2 + u_{st4}^2 + u_{st5}^2 + u_{st6}^2}$$

where u_c is the standard uncertainty.

2.5 Combined expanded uncertainty

The expanded uncertainty of the HbA2 reference materials was calculated via the following formula:

$$U = u_c \times k$$

where U is the expanded uncertainty. A coverage factor k of 2 corresponds to the 95% confidence level; therefore, the expanded uncertainty can be analyzed. The results of the associated uncertainties are shown in Table 2.

Supplementary Material 3

3. Stability study

3.1 Long-term stability

Thirty-six vials/level HbA2 reference materials were selected from all samples (stored at -70°C) by stratified random sampling to evaluate long-term stability; these samples were divided into 2 portions and then stored at -70°C or -20°C. Three vials/level control materials were tested 5 times/vial at the 1^{st}, 2^{nd}, 3^{rd}, 6^{th}, 9^{th}, 12^{th}, 18^{th}, and 24^{th} months for samples stored at -70°C and at the 0.5^{th}, 1^{st}, 2^{nd}, 3^{rd} and 6^{th} months for samples stored at -20°C.

3.2 Bottle-opening stability

Twelve vials/levels of HbA2 reference materials were selected from all samples (stored at -70°C) by stratified random sampling to assess bottle opening stability. Two vials/level HbA2 reference materials were opened on days 0, 1, 2, 3, 4, and 5. The samples were subsequently stored at 2–8°C. All HbA2 reference materials were tested 3 times/vial on the 5^{th} day.

3.3 Freeze–thaw stability

Twelve vials/level HbA2 reference materials were selected for the study of freeze–thaw stability via stratified random sampling from total samples (stored at -70°C). Two vials/level HbA2 reference materials were subjected to 1–5 freeze–5 freeze–thaw cycles between -40°C and 2°C~8°C on the 0, 1^{st}, 2^{nd}, 3^{rd}, 4^{th} and 5^{th} cycles. All HbA2 reference materials were tested 3 times/vial.

3.4 Cold storage stability

Eighteen vials of HbA2 reference material were used to evaluate the stability of cold storage via stratified random sampling of the total samples. Three vials of HbA2 reference material were selected on days 0, 1, 2, 3, 4 and 5 and then stored at 2–8°C. All the HbA2 reference materials were tested three times per vial on the fifth day.

3.5 Shipped stability

Eighteen vials of HbA2 reference material were selected by stratified random sampling from all samples stored at -70°C to study shipping stability. The vials were placed on a shaker to simulate transportation at -20°C. Three vials of HbA2 reference material per level were tested three times per vial on days 0, 1, 2, 3, 4 and 5.

3.6 Room temperature stability

Twelve vials of HbA2 reference material were selected by stratified random sampling from all the samples stored at -70°C. These vials were then stored at 18–25°C to assess room temperature stability. Two vials/level HbA2 reference materials were tested 3 times/vial at the 1st, 3rd, 6th, 9th, 12th, and 24th hours.

3.7 Statistical analysis

The stability data of the control materials were calculated via simple linear regression via SPSS 16.0. When $|b_1| \leq t_{0,95} \times s(\beta_1)$ were considered not statistically significant. All the results are shown in Table S5.

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