**Supplementary Material**

**Commutable whole blood reference materials for hemoglobin A1c validated on multiple clinical analyzers**

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**IDMS Procedure**

Four calibration blends were prepared by mixing calibration standard and isotope labelled standard solutions with the isotope molar ratios being close to 0.7, 0.85, 1.15 and 1.3, respectively. The isotope molar ratio in sample blend was controlled within the acceptable range of 0.9 to 1.1 with optimum value of 1 by spiking same isotope labelled standard solution into the sample. Isotope molar ratio obtained from weighing and the measured isotope ratio by LC-MS/MS should have a linear relationship (see Fig S1 below). The “isotope molar ratio vs measured isotope ratio” calibration curve with a goodness-of-fit for liner regression, R2, better than 0.999 was accepted for further calculation.



**Fig S1** Molar ratio versus isotope ratio in calibration blends and sample blends.

For the unknown sample, the isotope molar ratio can be calculated by the linear regression function:

|  |  |  |
| --- | --- | --- |
|  | | (1) |
| where | *RM* = isotope molar ratio in sample blend | |
|  | *RB* = isotope ratio in sample blend measured by LC-MS/MS | |
|  | *m* = slope of the linear regression plot based on the four calibration | |
|  | *b* = interception on *y* axis for the linear regression plot | |

Isotope molar ratio in sample (*RM*) is proportional to the concentration of the analyte in the sample (*CX*) as in Equation (2). The concentration of the analyte in the sample can be obtained from Equation (3) after rearrangement of Equation (2).

|  |  |  |
| --- | --- | --- |
|  | | (2) |
|  | | (3) |
| where | *CX* = molar fraction of analyte (µmol/g) in the sample | |
|  | *MX* = mass of the sample | |
|  | *MY* = mass of isotope labelled standard solution added to the sample | |
|  | *CY* = concentration of isotope labelled standard solution (µmol/g) added to the sample | |

According to ISO Guide 35:2006(E), for the linear fit of the four calibration blends, the gradient of the slope and the intercept on y axis can be expressed by Equations (4) and (5), respectively:

 (4)

 (5)

where: *RB i* = isotope ratio of each calibration blend measured by LC-MS/MS

= average of isotope ratio of the four calibration blends

*RM i* = isotope mass ratio of each calibration blend

= average of isotope mass ratio of the four calibration blends

Based on the preparation method of the calibration blends:

 (6)

 (7)

where: *MZi* = mass of the calibration standard solution for preparation of each calibration blend

*CZ* = concentration of calibration standard solution

*MYi* = mass of the isotope labelled standard solution for preparation of each calibration blend

*CY* = concentration of calibration standard solution

With the substitution using Equations (4) and (5), then using Equations (6) and (7), Equation (1) can be converted to Equation (8):



(8)

Let:



(9)

Equation (8) can be simplified as:

 (10)

In Equation (10), as *CZ* and *CY* are both constant, *RM’* is proportional to *RM* and also carried the same mathematical significance as *RM*. With the substitution using Equation (10), Equation (3) can be converted to Equation (11). As shown in Equation (9), *RM’* only consists of the measured isotope ratios and mass of solutions from balance weighing. Therefore, Equation (11) shows that *CX* is proportional to *CZ* but not affected by *CY*, and *CY*does not contribute to the measurement uncertainty of *CX*.

 (11)

**Amino acid analysis procedure**

Approximately 20 µL of each sample blend (VEc or GEc standard solution spiked with appropriate amount of isotope-labelled amino acid internal standards) with the concentration of about 0.04 to 0.05 µmol/g was pipetted into a 6 × 50 mm test tube, and the blend was freeze dried. The test tubes containing the lyophilized sample blends were placed into a hydrolysis vessel with 200 µL of 1% phenol in 6N HCl. The vessel was then vacuumed and purged with nitrogen for three times (ending with vacuum), and heated in an oven at 120 oC for 24 h, where the lyophilized sample blends reacted with the HCl vapor. After hydrolysis, the lyophilized blends were reconstituted with water and diluted to about 150 ng/g (amino acid concentration) for LC-MS/MS analysis.

**Sample preparation procedure**

To a 2 ml plastic centrifuge tube was added a calculated amount of 25 mM ammonium buffer solution (pH = 4). The blood sample (30 µL) was then weighed into the plastic centrifuge tube with the buffer solution and spiked with appropriate amount of either *d7*-VE for HbA0 measurement or *d7*-GE for HbA1c measurement. The total volume of buffer solution, blood sample and isotope-labelled standard solution was kept at 1.0 mL. After vigorous vortexing, the sample blend was allowed to equilibrate for 30 min at ambient temperature. 100 µL of the sample blend solution was then transferred to another 2 mL plastic centrifuge vial, and 200 µL of endoproteinase Glu-C aqueous solution (250 µg/mL) was added. The centrifuge vial was incubated at 37 oC for proteolysis in a water bath for 24 h. After incubation, the sample blend was diluted to about 500 ng/g (hexapeptide concentration) for LC-MS/MS analysis.

**LC-MS/MS settings**

For the analysis of amino acids in the determination of concentrations of VEc and GEc stock calibration standard solutions, the separation was performed using a Zorbax Eclipse AAA column (4.6 × 150 mm, 5 µm) with a binary gradient consisting of mobile phase A (0.1% trifluoroacetic acid in water) and mobile phase B (0.1% trifluoroacetic acid in acetonitrile). The settings are listed in Table S1.

**Table S1.** LC-MS/MS settings of amino acid analysis.

**HPLC conditions**

|  |  |  |  |
| --- | --- | --- | --- |
| Time (min) | Module | Events | Parameter |
| 0.00 | Pumps | Pump B Conc. | 10 |
| 5.00 | Pumps | Pump B Conc. | 10 |
| 10.00 | Pumps | Pump B Conc. | 30 |
| 14.00 | Pumps | Pump B Conc. | 30 |
| 14.50 | Pumps | Pump B Conc. | 10 |
| 16.00 | System Controller | Stop |  |

**MS Parameter**

|  |  |
| --- | --- |
| Events | Parameter |
| Scan Type | MRM |
| Polarity | Positive |
| Resolution Q1 | Unit |
| Resolution Q3 | Unit |
| CUR | 20.00 |
| IS | 5000.00 |
| TEM | 450.00 |
| GS1 | 45.00 |
| GS2 | 45.00 |
| CAD | Medium |

**MRM Transitions**

|  |  |
| --- | --- |
| L-leucine | 132/86 |
| 1,2-13C2-L-leucine | 134/87 |
| L-proline | 116/70 |
| 13C5,15N-L-proline | 122/75 |

In the determinations of HbA0 and HbA1c peptides (VE and GE) in blood samples, an Agilent Zorbax Eclipse Plus C18 column was used for the separation with a binary gradient consisting of mobile phase A (0.1% formic acid for hemolysate sample) and mobile phase B (acetonitrile). The LC-MS/MS settings are listed in Table S2.

**Table S2** LC-MS/MS settings for VE and GE in blood samples

**HPLC conditions**

|  |  |  |  |
| --- | --- | --- | --- |
| Time | Module | Events | Parameter |
| 0.10 | Pumps | Pump B Conc. | 5 |
| 4.00 | Pumps | Pump B Conc. | 60 |
| 4.50 | Pumps | Pump B Conc. | 60 |
| 6.00 | Pumps | Pump B Conc. | 90 |
| 6.50 | Pumps | Pump B Conc. | 5 |
| 7.50 | System Controller | Stop |  |

**MS Parameter**

|  |  |
| --- | --- |
| Events | Parameter |
| Scan Type | MRM |
| Polarity | Positive |
| Resolution Q1 | Unit |
| Resolution Q3 | Unit |
| CUR | 20.00 |
| IS | 4500.00 |
| TEM | 450.00 |
| GS1 | 45.00 |
| GS2 | 45.00 |
| CAD | Medium |

**MRM Transitions**

|  |  |
| --- | --- |
| VE | 695.4/350.2 |
| *d*7-VE | 702.5/357.2 |
| GE | 857.5/458.0 |
| *d*7-GE | 864.7/465.0 |

**Homogeneity Results.**

**Table S3.** Results of homogeneity testing (ANOVA) for Level 1 of the blood CRMs.

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Vial No. | Peak Area Ratio VE/GE | | MSbv | MSwv | F | F (critical) | P Value | Conclusion |
| Sub-sample 1 | Sub-sample 2 |
| 1 | 101.030 | 102.629 | 1.253 | 1.640 | 0.764 | 2.854 | 0.660 | Homogeneous |
| 2 | 101.531 | 103.363 |  | | | | | |
| 3 | 102.401 | 103.444 |
| 4 | 104.238 | 102.556 |
| 5 | 102.929 | 102.722 |
| 6 | 99.576 | 102.013 |
| 7 | 101.820 | 104.666 |
| 8 | 102.059 | 104.667 |
| 9 | 101.304 | 103.586 |
| 10 | 102.086 | 101.722 |
| 11 | 103.029 | 102.863 |

**Table S4.** Results of homogeneity testing (ANOVA) for Level 2 of the blood CRMs.

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Vial No. | Peak Area Ratio VE/GE | | MSbv | MSwv | F | F (critical) | P Value | Conclusion |
| Sub-sample 1 | Sub-sample 2 |
| 1 | 77.687 | 79.293 | 0.555 | 0.698 | 0.796 | 2.854 | 0.636 | Homogeneous |
| 2 | 78.173 | 77.011 |  | | | | | |
| 3 | 78.199 | 77.833 |
| 4 | 78.689 | 77.388 |
| 5 | 77.744 | 78.658 |
| 6 | 78.396 | 79.390 |
| 7 | 77.583 | 77.576 |
| 8 | 78.818 | 76.423 |
| 9 | 77.868 | 78.570 |
| 10 | 78.357 | 77.821 |
| 11 | 77.481 | 76.361 |

**Table S5.** Results of homogeneity testing (ANOVA) for Level 3 of the blood CRMs.

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Vial No. | Peak Area Ratio VE/GE | | MSbv | MSwv | F | F (critical) | P Value | Conclusion |
| Sub-sample 1 | Sub-sample 2 |
| 1 | 84.775 | 84.627 | 0.387 | 0.366 | 1.058 | 2.854 | 0.460 | Homogeneous |
| 2 | 86.181 | 85.771 |  | | | | | |
| 3 | 84.530 | 85.693 |
| 4 | 85.576 | 84.422 |
| 5 | 84.758 | 85.009 |
| 6 | 84.737 | 84.151 |
| 7 | 85.151 | 85.442 |
| 8 | 85.493 | 84.516 |
| 9 | 85.091 | 84.756 |
| 10 | 83.926 | 85.435 |

**Stability results**

**Table S6.** Results of stability testing for blood materials in 2016 EQA.

|  |  |
| --- | --- |
|  | **Material 1** |
| Occassion 1, day 0 (mmol/mol) | 37.1 |
| Occassion 2, day 33 (mmol/mol) | 38.5 |
| Occassion 3, day 43 (mmol/mol) | 38.0 |
| Occassion 4, day 406 (mmol/mol) | 36.7 |
| Slope (b1, mmol/mol / day) | -0.0028 |
| Standard uncertainty of b1 [u(b1)] (mmol/mol / day) | 0.0024 |
| Significance of b1  |b1/u(b1)|, *tcal* | 1.20 |
| *tcritical* | 4.30 |
| Conclusion | Stable, *tcal* < *tcritical* |

|  |  |
| --- | --- |
|  | **Material 2** |
| Occassion 1, day 0 (mmol/mol) | 57.0 |
| Occassion 2, day 28 (mmol/mol) | 58.1 |
| Occassion 3, day 57 (mmol/mol) | 57.9 |
| Occassion 4, day 280 (mmol/mol) | 57.1 |
| Slope (b1, mmol/mol / day) | -0.0018 |
| Standard uncertainty of b1 [u(b1)] (mmol/mol / day) | 0.0028 |
| Significance of b1  |b1/u(b1)|, *tcal* | 0.65 |
| *tcritical* | 4.30 |
| Conclusion | Stable, *tcal* < *tcritical* |

**Table S7.** Results of stability monitoring of blood CRMs.

|  |  |  |  |
| --- | --- | --- | --- |
|  | Level 1 | Level 2 | Level 3 |
| Certified Value, *xCRM* (mmol/mol) | 35.1 | 50.3 | 65.8 |
| Standard uncertainty of certified value (*k* = 2),  *uCRM* (mmol/mol) | 1.0 | 0.95 | 1.3 |
| Time of stability monitoring after value assignement (days) | 325 | 325 | 228 |
| Stability monitoring value, *xmon* (mmol/mol) | 36.7 | 52.1 | 67.7 |
| Standard uncertainty of stability monitoring value, *umon* (mmol/mol) | 0.10 | 0.41 | 0.13 |
| |*xCRM - xmon*| (mmol/mol) | 1.6 | 1.8 | 1.9 |
|  | 2.0 | 2.1 | 2.6 |
| Conclusion | Stable,  |*xCRM - xmon*| < | Stable,  |*xCRM - xmon*| < | Stable,  |*xCRM - xmon*| < |