CCQM-K177
Determination of Growth hormone in serum using isotope dilution mass spectrometry

Final Report
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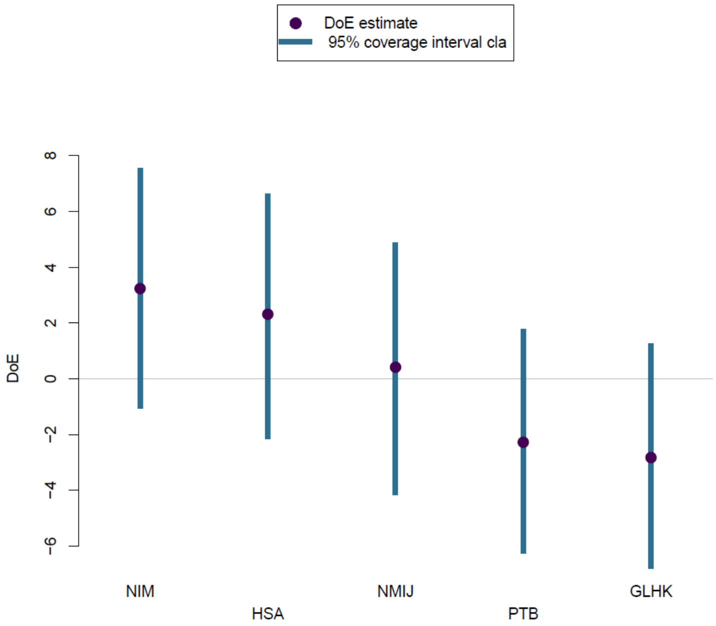
Abstract

This comparative measurement aims to demonstrate the ability to determine the amount of substance of a small protein in a biological matrix such as blood serum in the concentration range around 0.50 pmol/g (0.23-0.90 pmol/g) using isotope dilution mass spectrometry. Growth hormone (GH) was selected as the model protein, with the measurand being total GH in serum. A pooled serum sample from blood donors was used as study material. Five National Metrology Institutes (NMIs) participated and submitted results, all of which were included in the calculation of the Key Comparison Reference Value (KCRV). All participants agreed to use the DerSimonian and Laird method to establish the consensus value. Due to greater-than-expected variability among participants compared to the uncertainties reported by their laboratories, an excess measurement uncertainty (referred to as "dark uncertainty") was estimated. This value provided a quantitative assessment of previously unidentified uncertainty components. The DerSimonian and Laird consensus value was determined to be 11.79 ± 0.99 ng/g, with the dark uncertainty (τ) estimated at 2.09 ng/g.

Graphical Abstract



Unilateral Degrees of Equivalence

Lab	DoE.x	DoE.U95	DoE.Lwr	DoE.Upr
NIM	3.23	4.23	-0.981	7.47
HSA	2.31	4.31	-2.08	6.55
NMIJ	0.412	4.43	-4.09	4.8
PTB	-2.28	3.91	-6.16	1.68
GLHK	-2.83	3.95	-6.73	1.16

Content

BACKGROUND

During the PAWG - video conference in April 2021, the group decided to proceed with a key Track A study on the determination of growth hormone (GH) in a pooled human blood serum sample. The small protein, containing 191 amino acids and smaller isoforms, serve as a model for the determination of low-abundant proteins in serum.

OBJECTIVES

This comparison enables the participating NMIs/DIs to demonstrate their measurement capabilities in quantification of a protein located in section D of the matrix model of the PAWG with a molar mass ≤ 100 kDa in a biological fluid at a concentration range around 0.50 pmol/g (0.23-0.90 pmol/g).

MEASURANDS

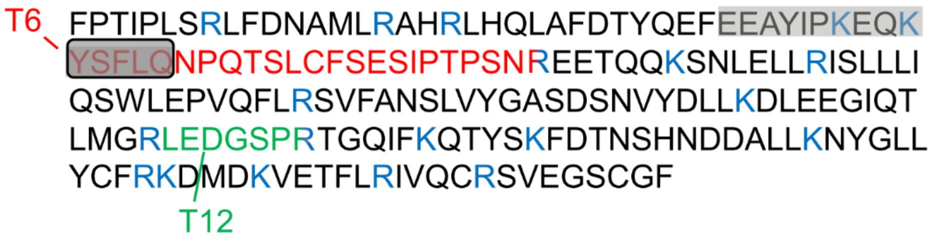
The measurand for the comparison is total-GH in serum at physiological levels. Optionally, the capability of isoform-differential determination of GH can be demonstrated by quantifying 22 kDa-GH or other single isoforms as well as total-GH. The isoforms can be differentiated based on small differences in their primary structure. The difference between the 20 kDa- and 22 kDa-GH is shown in Figure 1.

In this comparison, participants were asked to submit results for total-GH and optionally also for 22 kDa-GH. For this purpose, 'signature peptides' can be used as surrogate measurands. Two of them, T6 (red) and T12 (green), are highlighted in Figure 2. These are generated by enzymatic cleavage of the molecule when using trypsin as a protease. However, T6 selectively probes 22 kDa-GH, while excluding the 20 kDa form. This results from the knowledge that the first five amino acids of T6 (grey box in Figure 2) are absent in the 20 kDa GH. In contrast, T12 can be taken as measuring total GH since this peptide sequence is observed in both the 20 and 22 kDa forms of GH.

Participants were requested to measure the amount of substance concentration of the T6 and T12 peptides if using an enzymatic digestion approach. Other tryptic fragments could also be reported if assessed to be specific to the measurand.


FPTIPLSRLFDNAMLRAHRLHQLAFDTYQEFEEAYIPKEQK
YSFLQNPQTSLCFSESIPTPSNREETQQKSNLELLRISLLLI
QSWLEPVQFLRSVFANSLVYGASDSNVYDLLKDLEEGIQT
LMGRLEDGSPRTGQIFKQTYSKFDTNSHNDDALLKNYGLL
YCFRKDMDKVETFLRIVQCRSVEGSCGF

Figure 1: Primary structure of 22 kDa-GH. The sequence stretch highlighted in grey is absent in the 20 kDa-GH isoform.



FPTIPLSRLFDNAMLRAHRLHQLAFDTYQEFEEAYIPKEQK
YSFLQNPQTSLCFSESIPTPSNREETQQKSNLELLRISLLLI
QSWLEPVQFLRSVFANSLVYGASDSNVYDLLKDLEEGIQT
LMGRLEDGSPRTGQIFKQTYSKFDTNSHNDDALLKNYGLL
YCFRKDMDKVETFLRIVQCRSVEGSCGF

Figure 2: Primary structure of 22 kDa GH. Signature peptides T6 and T12 are highlighted in red and green, respectively. Tryptic cleavage points (behind each K or R) are highlighted in blue. The first five amino acids of T6 (grey box) are absent in the 20 kDa GH.

Study materials

The material prepared for this key comparison is a frozen pooled human serum. The serum pool was prepared in the laboratory of Physikalisch-Technische Bundesanstalt (PTB) from blood samples of male and female donors. The samples were obtained from a blood bank (DRK-Blutspendedienst NSTOB, Springe, Germany) and from Laboratorium Klin. Chemie & Haematologie University Medical Center Utrecht, Netherlands. The quantity of GH in the final serum pool was tested using an electrochemiluminescence immunoassay (ECLIA) in a routine laboratory (LADR, Geesthacht, Germany) and found to match the prerequisite for the preparation of a material containing GH in the range of 5-20 ng/g. Additional immunoassays: Immulite 2000 (Siemens) , iSYS (DSL) and Liaison (Diasorin) were used for the determination of GH in the material. The measurements were carried out in Laboratorium Klin. Chemie & Haematologie University Medical Center Utrecht, Netherlands. The material was re-packed in plastic tubes containing 3 mL aliquots of serum each.

Participants were also provided with aliquots of stock solutions of GH reference material and U-^{15}N-labeled 22 kDa-GH. The mass fraction of GH reference material and U-^{15}N-labeled 22 kDa-GH in stock solutions was 6.57 and 6.54 ng/mg, respectively. Both stock solutions of GH had to be used by study-participants for the calibration of GH-measurement. For this purpose, both solutions had to be further diluted in blank serum to adjust the target GH-levels of the study material.

For the gravimetric preparation of GH stock solution, an aliquot of blank serum was spiked with a defined amount of GH. As blank serum a pool (SeraCon II) was used, which was obtained from SeraCare, Milford, USA. This serum pool was tested using an *electrochemiluminescence*-immunoassay (ECLIA, Roche Diagnostics AG) in a routine testing laboratory (LADR, Geesthacht, Germany) and GH was found, as expected, at a trace-level (0.08 ng/g).

For the standard addition of GH, a solution of 22 kDa-GH (International Standard 98/574) in water and acetonitrile (1/1, v/v) was prepared and value-assigned by amino acid analysis using ID-MS. In detail, results obtained from analysis of phenylalanine, valine, leucine, isoleucine and proline were 45.53, 45.98, 44.86, 44.56 and 45.57 nmol/g, respectively. The concentration of 22 kDa-GH was determined to be 45.30 nmol/g (mean of results from amino acids). The expanded measurement uncertainty (U_{rel}) of this SI-traceable value-assignment was estimated to be 2.2 % at the 95 %-confidence level.

The purity of 22 kDa-GH was checked by direct infusion and high-resolution mass spectrometry. No peptide or protein related impurities were identified, which could interfere with the amino acid analysis. The expected primary structure of recombinant 22 kDa-GH was confirmed by peptide mapping of a tryptic digest using liquid chromatography coupled to mass spectrometry (LC-MS). The molar mass of 22124.8 g/mol was calculated according to the defined primary structure of 22 kDa-GH and reported to study participants with an estimation of measurement uncertainty.

Aliquots of GH stock solutions were packaged in polypropylene vials. Each vial was filled with 150 µL of either stock solution of GH reference material or U-^{15}N-22 kDa-GH.

HOMOGENEITY

The homogeneity of study material was assessed using ECLIA. Twelve aliquots, each containing 1 mL of serum material, was randomly selected, using a stratified sampling model and three subsamples were taken from each aliquot. Results are shown in Figure 3.

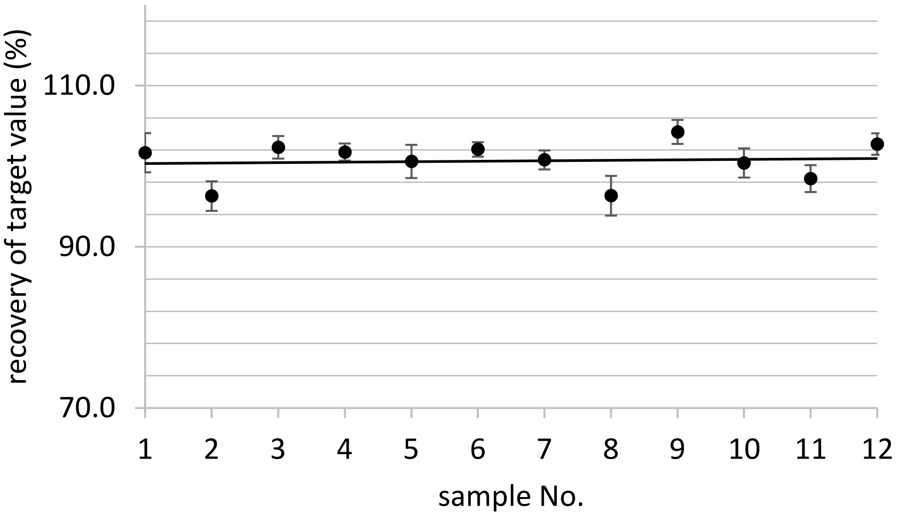


Figure 3: Results obtained for aliquots of study material from replicate determinations of GH using ECLIA.

Analysis of variances (ANOVA) was used for data evaluation which enables separation of the variation between aliquots (*sbb*) from variation within aliquots. Summary of the ANOVA at 95% confidence level is shown in Table 1.

Table 1: Summary of ANOVA for GH

Source of variance	*SS*	DF	MS	F	p-Value	$F_{critical}$
Between aliquots	201.209	11	18,292	4.196	0.16%	2.220
Within aliquots	104.636	24	4.360			
Total	305,845	35	8.738			

The standard uncertainty of inhomogeneity (*ubb*) was calculated using equation 1 in accordance with ISO Guide 35: Reference materials – General and

statistical principles for certification, Geneva, Switzerland, 2006.

$$ubb = \sqrt{\frac{MSwithin}{n}} \times \sqrt[4]{\frac{2}{v_{MSwithin}}} \qquad (1)$$

where:

ubb = standard uncertainty of inhomogeneity

MSwithin = mean square within-aliquot variance

v*MSwithin* = degree of freedom of MS_{within}
n = number of measurement replicates per aliquot (n=3)

The relative standard uncertainty of inhomogeneity (*ubb*) was 0.65 % for GH in study sample. The F-value was higher than the critical value indicating a low significance of between-aliquot heterogeneity, which was not masked by the method-repeatability of ECLIA ($\sqrt{MSwithin}$). Therefore, in addition to the standard uncertainty of inhomogeneity (*ubb*), the between-aliquot variation (*sbb*) was calculated using equation 2.

$$sbb = \sqrt{\frac{abs(MSbetween - MSwithin)}{n}} \qquad (2)$$

where:

sbb = between-aliquot variation

MSbetween = mean square between-aliquot variance

MSwithin = mean square within-aliquot variance
n = number of replicates per aliquot (n=3)
Between-aliquot variation (*sbb*) and method-repeatability was 2.14 and 2.08 %, respectively. The study material was further tested if it is sufficiently homogeneous

for the measurement comparison. For this purpose, nine aliquots were analyzed in the laboratory of study coordinators at PTB using ID-MS. The mass fraction of total-GH was found to be 9.32±0.28 ng/g. The CV-value of 3.0% did not reveal any inconsistencies. Therefore, the study material was comprehended as sufficiently homogeneous for the subsequent comparison of measurement capability.

STABILITY

The stability of the study material was assessed at different temperatures (-20, +5, and +25 °C) using an isochronous design. The same sample-size and test-method (ECLIA) as described in the homogeneity study was used. In general, two randomly selected aliquots were kept at the selected temperatures, then transferred to the reference temperature of -78 °C and stored until analysis. At a temperature of -80 ± 5 °C, GH was found to be stable in human serum for >10 years (Wagner IV et al., Eur J Endocrinol, 2014, 171, 389–397).

The stability at -20 °C was evaluated over a long-term period of 40 weeks. Samples were exposed to -20 °C for 1, 2, 4, 8, 16, 24, 32 and 40 weeks.

The stability at +5 °C was evaluated over a period of 4 weeks. Samples were kept at +5 °C for 1, 2 and 4 weeks.

For evaluating the stability at +25 °C, samples were kept at +25 °C for 1, 2, 4, 24 and 48 hours. All samples were stored at the reference temperature of -78 °C until analysis in the laboratory LADR, Geesthacht, Germany. Two subsamples were taken from each aliquot for GH-determination. Using a Student’s *t*-test on the slope of the linear regression at 95 % level of confidence, significant instability of GH in the study material was not observed at any tested temperature. However, it was recommended to store materials at -20 °C immediately upon receipt. Linear regression curves of GH recovery after exposing the study sample to different temperatures are shown in Figure 4. Stability data are summarized in Table 2.

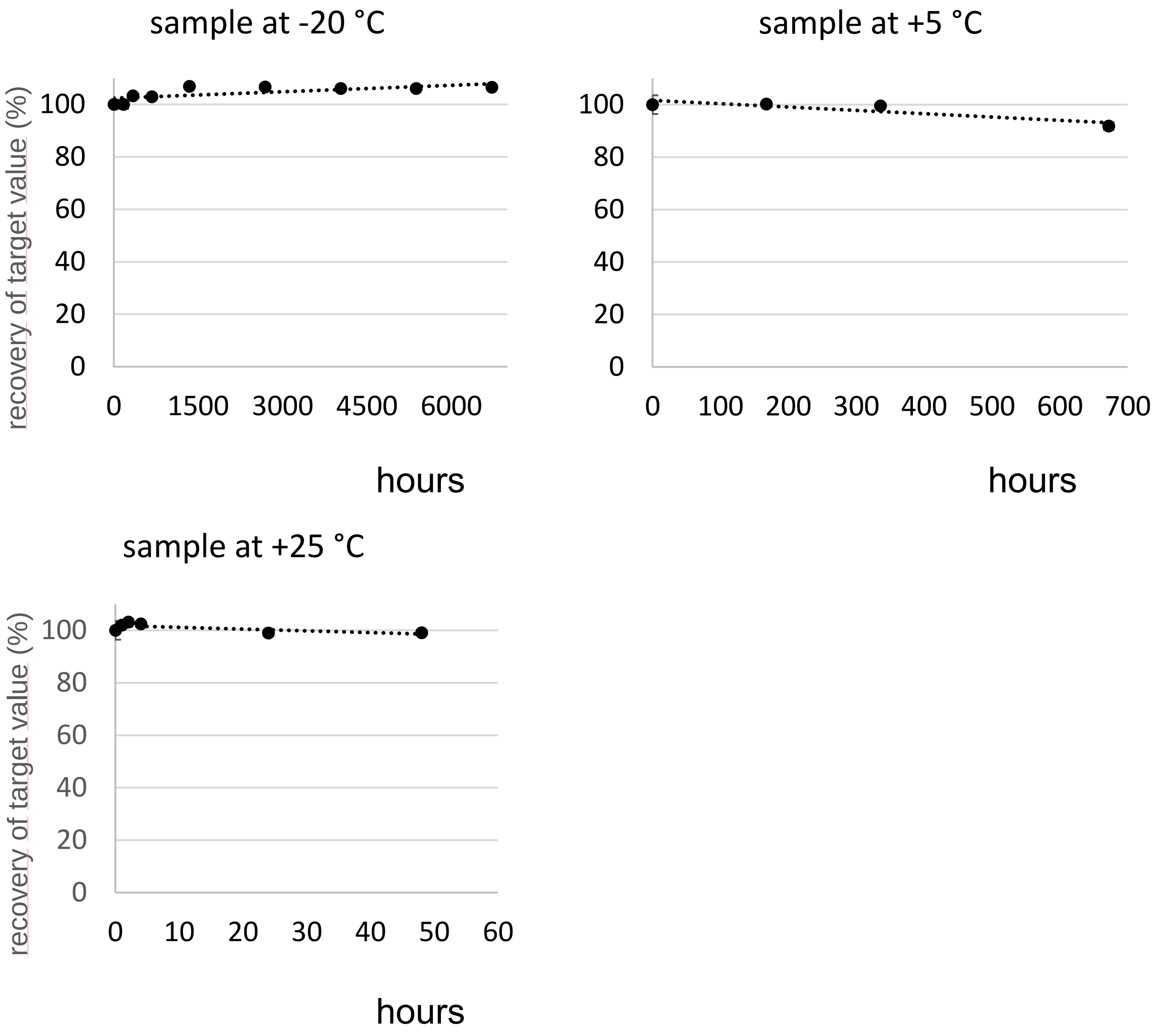


Figure 4: Linear regression curves of GH recovery after exposing the study sample to -20, +5 and + 25 °C.

Table 2: Summary of stability study for GH in study sample at -20, +5 and +25 °C

Descriptions	stability test at -20°C	stability test at +5°C	stability test at +25°C
Slope of the regression line (b)	0.0008	-0.0127	-0.0667
Intercept of the regression line (b_0)	102.38[1]	101.63[1]	101.80[1]
Variance of the points (s^2)	7.94	16.42	3.27
Standard deviation of the points (s)	2.82	4.05	1.81
Uncertainty of slope [$s(b)$]	0.0003	0.0044	0.0330
Calculated t ($\frac{\lvert b \rvert}{s(b)}$)	2.67	2.89	2.02
Critical t factor ($t_{0.95,n-2}$)	2.365	4.30	2.78

[1] Recovery of target value (%)

From the uncertainty of slope $s(b)$, the relative standard uncertainty of instability at -20 °C was estimated to be below 1.0 % for the study sample. The study material was found to be sufficiently stable under transport conditions and time schedule for analysis.

Instructions and Participants

The participating institutes were requested to report their results as the mass fraction of total-GH in serum in the unit of ng/g for at least three aliquots of study material. Participants were requested to calculate one overall result and report this with an estimation of its combined standard uncertainty. The measurement procedure including the calculation of results had to be described and a budget for the estimation of measurement uncertainty at the 95% confidence level had to be provided. The timeline of the comparison is shown in Table 3.

Table 3: Schedule for the comparison

Date	Event
December 2021	Call for participation
February 2022	Distribution of study materials was started
September 2022	Deadline for submission of results
January 2023	Presentation of results at the PAWG meeting
April 2023	Presentation of the Draft A report

A total of 7 NMIs/DIs registered to participate in the comparison for determination of GH in the study CCQM-K177. Each participant was provided with one vial of study material.

Most of the participants received the study material intact. Participants from INMETRO, Brazil were not able to receive materials due to customs issues in Brazil. Some precipitation was observed in sample which was sent to NIM, China. The other participants reported that samples were received in a frozen state. Information on participating NMIs/DIs, contacts and sample receipts are summarized in Table 4.

Table 4: Information on participating NMIs/DIs, contacts and sample receipts

No.	**Participating Institutes**	**Economy**	**Contact Person**	**Sample shipping date**	**Sample Receipt Date**
1	INMETRO Instituto Nacional de Metrologia, Qualidade e Tecnologia	Brazil	Paulo Beltrao	07 May 2022	-
2	Health Sciences Authority (HSA), Singapore	Singapore	Qinde Liu	07 Feb 2022	10 Feb 2022
3	NIM National Institute of Metrology, China	China	Youxun Jin	14 Feb 2022	24 Feb 2022
4	NMIJ National Metrology Institute of Japan	Japan	Tomoya Kinumi	07 Feb 2022	09 Feb 2022
5	National Research Council of Canada	Canada	Jeremy Melanson	07 Feb 2022	11 Feb 2022
6	PTB Physikalisch-Technische Bundesanstalt	Germany	Cristian Arsene	-	-
7	Pharmaceutical Chemistry Section, Analytical and Advisory Services Division Government Laboratory, HKSAR	Hong Kong, China	L H Tong	14 Feb 2022	19 Feb 2022

Results

A graph of the reported results is shown in Figure 5 and results are summarized, with some statistical data, in Table 5.

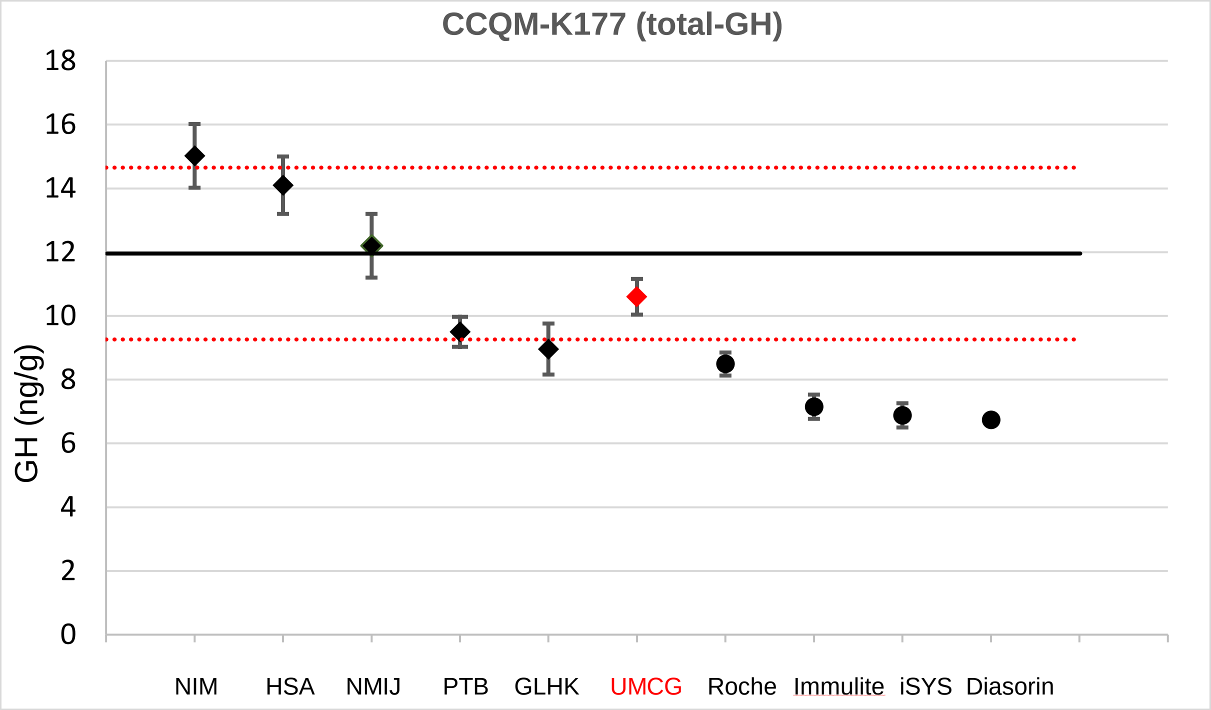


Figure 5: Reported mass fractions of GH in serum with combined standard uncertainties (*k*=1). The mean and the standard deviation *s* of results is shown as black and red lines, respectively.

The result of the Department of Laboratory Medicine (UMCG), Groningen, Netherlands and results of immunoassays Roche, Immulite, iSYS and Diasorin are only informative. They were not used for calculation of statistics.

Table 5: Summary of results for GH

NMI	No. of aliquots	Mean (ng/g)	Combined standard uncertainty (ng/g)	Expanded uncertainty[1] (ng/g)	Coverage factor[1], *k*
		Results for total-GH			
NIM	4	15.02	0.98	1.96	2.00
HSA	4	14.1	0.82	1.89	2.31
NMIJ	3	12.2	1.1	2.1	2.00
PTB	6	9.51	0.24	0.48	2.00
GLHK	3	8.96	0.40	0.80	2.00
UMCG[2]	3	10.6	0.24	0.48	2.00
$\bar{x}$		11.96			
s		2.69			
CV		22.5 %			
median		12.20			
		Results for 22 kDa-GH			
NIM	4	14.26	0.67	1.34	2.00
HSA	4	12.94	0.77	1.83	2.36
PTB	6	8.13	0.21	0.43	2.00
UMCG[2]	3	9.66	0.23	0.46	2.00
$\bar{x}$		11.25			
s		2.84			
CV		25.2 %			
median		11.30			

[1] 95 % level of confidence

[2] Department of Laboratory Medicine (UMCG), Groningen, Netherlands

$\bar{x}$ = *mean; s* = standard deviation; CV = $100 \cdot s/\bar{x}$; statistics for GH was calculated without the result of UMCG

A graph of the reported results and the DerSimonian and Laird value as consensus value with the associated standard uncertainties was plotted using the NIST Consensus Builder (NICOB), accessed through: NIST Consensus Builder on 28.06.2024. It is shown in Figure 6. The absolute and relative difference of each reported result from mean value ($D_i = w_i - w_{DSL}$ and $relative\ D_i = (w_i - w_{DSL}) * 100/w_{DSL}$) is plotted in Figure 7.

The expanded uncertainty (*U*), associated with the difference of each result from consensus value, was calculated using the NICOB. Parametric bootstrapping with 10000 replicates was used for uncertainty evaluation. All results are summarized in Tables 6 and 7.

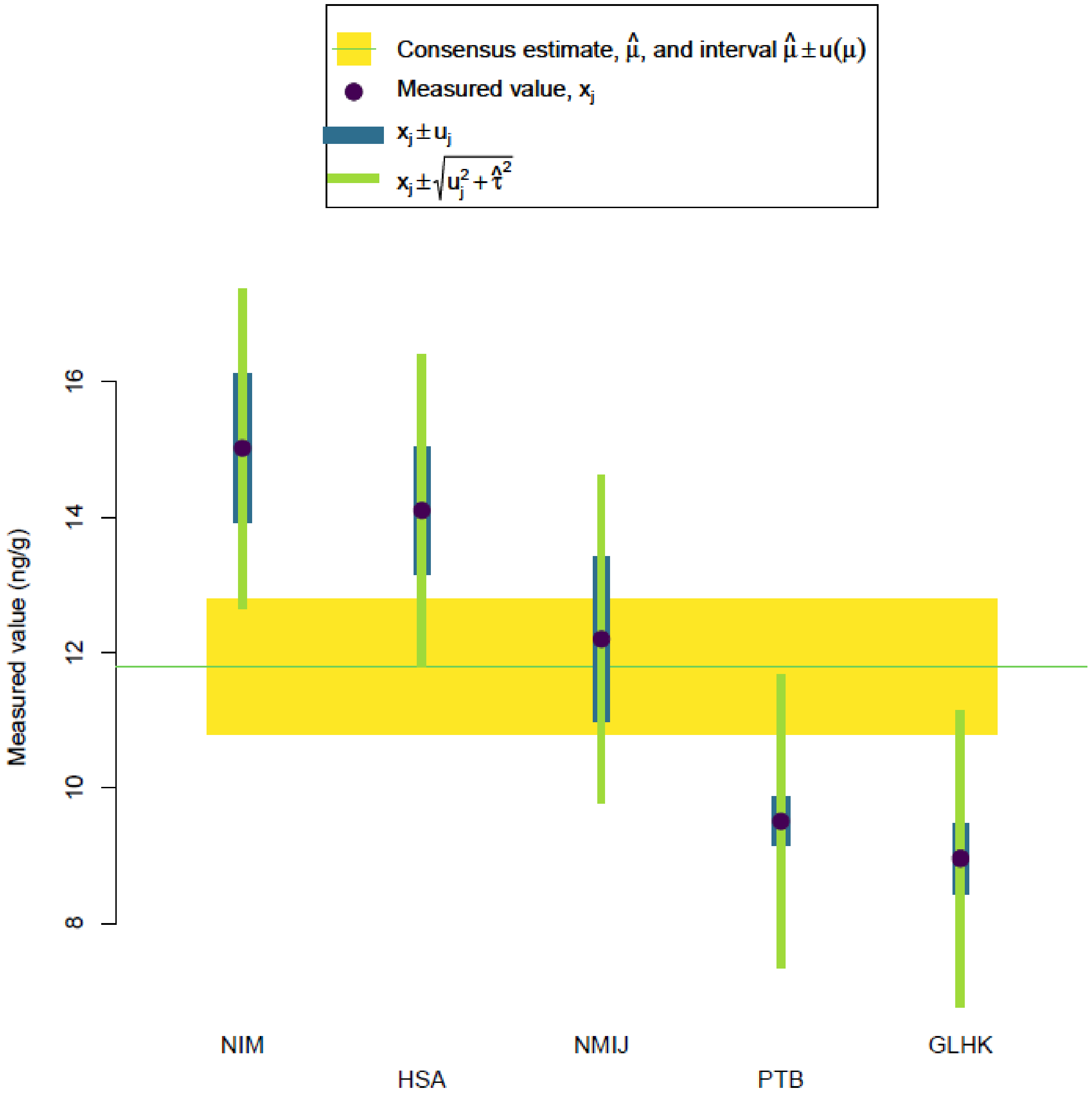


Figure 6: Reported mass fractions of GH in serum. Combined standard uncertainties (*k*=1) with and without considering dark uncertainty (τ=2.09 ng/g) are shown as green and blue error bars, respectively. The DerSimonian and Laird value (11.79 ng/g) and its associated uncertainty (0.99 ng/g) is shown as green line and yellow area.

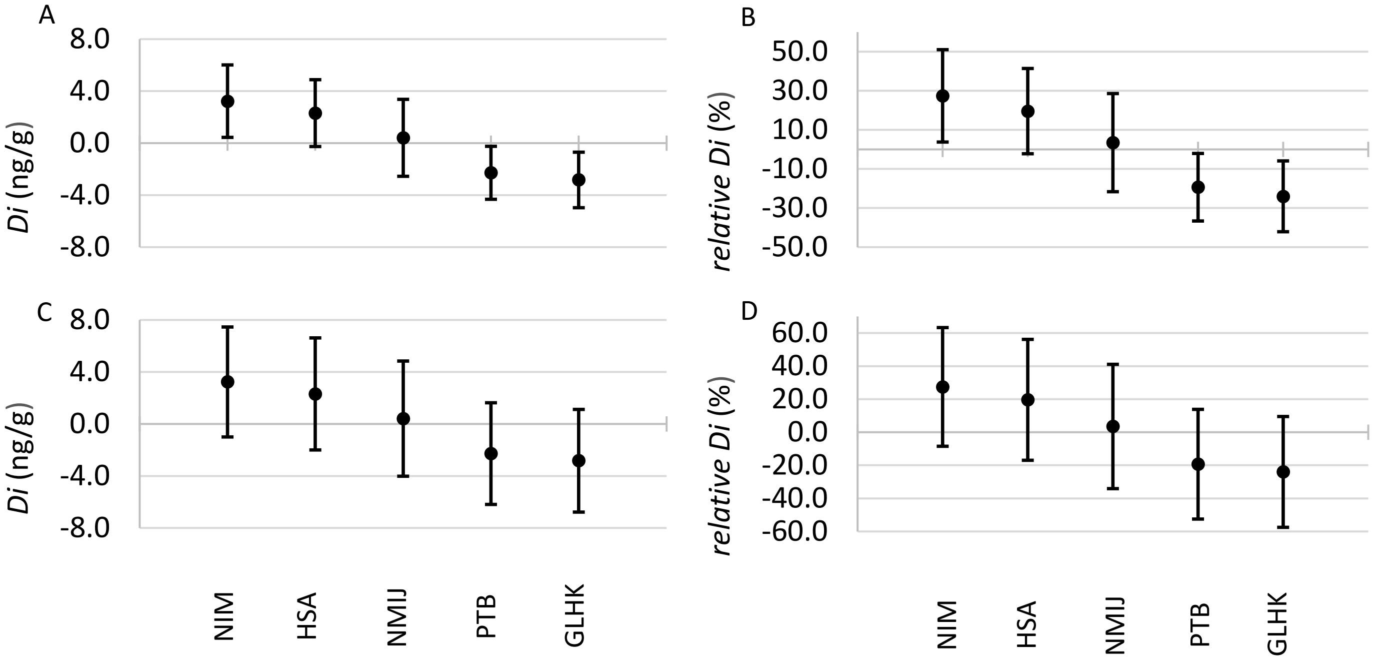


Figure 7 Absolute and relative difference of the reported mass fraction for total-GH in serum from the DerSimonian and Laird value (w_{DSL}: 11.79 ± 0.99 ng/g) without τ (A and B) and with τ (C and D)

Table 6: Degrees of equivalence with the DerSimonian and Laird value as KCRV

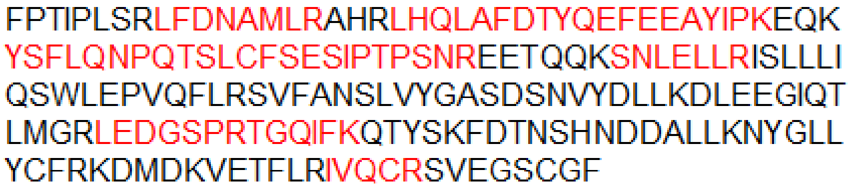
Participant	Di	U_{Di} with τ	U_{Di} without τ	Rel. Di	$Urel_{Di}$ with τ	$Di/U_{(Di)}$ with τ	$Di/U_{(Di)}$ without τ
NIM	3.23	4.23	2.79	27.40	35.88	0.76	1.16
HSA	2.31	4.31	2.57	19.59	36.56	0.54	0.90
NMIJ	0.41	4.43	2.96	3.48	37.57	0.09	0.14
PTB	-2.28	3.91	2.04	-19.34	33.16	-0.58	-1.12
GLHK	-2.83	3.95	2.14	-24.00	33.50	-0.72	-1.33

Table 7: Individual laboratory uncertainties

Participant	**x**	**u**	**nu**	**ut**
NIM	15.02	0.98	60	2.31
HSA	14.10	0.82	8	2.25
NMIJ	12.20	1.10	60	2.36
PTB	9.51	0.24	60	2.10
GLHK	8.96	0.40	60	2.13

x= measured values (ng/g), u=standard uncertainties (ng/g), nu=number of degrees of freedom, ut= standard uncertainties including dark uncertainty (τ)

The study participants used several signature peptides for GH determination (T2, T4, T6, T8, T12, T13 and T20), as shown in Figure 8. These signature peptides allowed for a sequence-coverage of 39 %.



FPTIPLSRLFDNAMLRAHRLHQLAFDTYQEFEEAYIPKEQK
YSFLQNPQTSLCFSESIPTPSNREETQQKSNLELLRISLLLI
QSWLEPVQFLRSVFANSLVYGASDSNVYDLLKDLEEGIQT
LMGRLEDGSPRTGQIFKQTYSKFDTNSHNDDALLKNYGLL
YCFRKDMDKVETFLRIVQCRSVEGSCGF

Figure 8: Primary structure of 22 kDa-GH. Red highlighted sequence stretches emphasize the signature peptides, targeted by the participants.

SUMMARY OF TECHNICAL INFORMATION

In Table 8 the technical information, which was provided in the “Report of Results Form” by participating NMIs/DIs, is summarized. All participants used double isotope-dilution mass spectrometry for determination of signature peptides of GH after tryptic digestion. The coordinating laboratory prepared a stock solution of the GH reference material (International Standard 98/574) and another stock-solution of U-^{15}N-22 kDa-GH in blank serum. Aliquots of these stock solutions were shipped to each participant with the study material and were diluted to appropriate GH-levels by study-participants and used for the calibration of GH-measurement. Therefore, this study does not include any variation for the source of the calibration material used by each laboratory.

Table 8: Summary of technical information

NMI/DI	Targeted signature peptide of GH	Description of sample pre-treatment & measurement
NIM	T2, T4, T6, T8, and T12	1. Standard Preparation. A stock standard of GH (297.17 fmol/mg, 6.57 ng/mg) and labeled GH (292.14 fmol/mg, 6.54 ng/mg) in serum was diluted 22 fold with blank serum under gravimetric control. The diluted serum was gently mixed for 1 h before the preparation of calibrators and samples. 2. Sample and Calibrator Preparation. 500 µL of serum was taken and mixed with the labeled GH. The calibrator consisted of 500 µL of blank serum with the GH standard at a concentration of about 14.0 ng/g and labeled GH at the same concentration. The amount of the labeled protein was equivalent to GH in both calibrators and samples. 3. Tryptic Digestion Sample and calibrator were incubated at 37 °C while gently shaking. 65 µL of a TRIS-solution consisting of 302.5 mg of TRIS (base) and 395 mg of TRIS•HCl in 1 mL of water was added to 500 µL serum (samples or calibrators, resp.). 50 µL of trypsin solution (12 mg of trypsin dissolved in 1.8 mL of 50 mM acetic acid) was added dropwise. Then, 100 µL of acetonitrile was added dropwise every 30 minutes for 11 times, and 50 µL of trypsin solution was added at 30, 90, 150, 210, and 270 minutes. The incubation was continued over night. 3.2 mg of dithiothreitol was added, and the calibrators and samples were gently shaken at 37 °C for 1 hour. The precipitate was spun down for 2 × 10 minutes at 17,860 g. After the first centrifugation, the supernatant was separated from the precipitate, and the remaining pellet was washed with 1 mL of water/acetonitrile (50/50, v/v). After vigorous vortexing and ultrasonic treatment for 1 minute, vortex again. After the second centrifugation, the ‘wash solution’ was added to the liquid phase from the first centrifugation. All the collected liquid was dried at 20 °C under 20 mbar. The dried residue was extracted with 400 µL of water. After vortexing, 100 µL of TFA (16%) was added and vortexed again. A 10-minute centrifugation at 3,960 g was performed, and the supernatant was separated. The remaining pellet was washed with 300 µL of water. After vortexing and the second centrifugation at 3,960 g for 10 minutes, the supernatant was added to the liquid phase obtained in the previous step. 4. Sample Cleanup. Two fold liquid chromatography was employed for extraction of targeted signature peptides after serum trypsin digest. Step 1 ('pH 2 RP-LC clean-up'): The column Jupiter Proteo (RP-C12, 90Å, 4 µm, 250 × 10 mm) was used. Mobile phase A: water (0.1% CF_3COOH, v/v); mobile phase B: acetonitrile (0.1% CF_3COOH, v/v). The flowrate was set at 2 mL/min and the chromatographic separation was performed at 25 ^{o}C. The elution gradient was as follows: 0-5 min, 1% B; 5-50 min, 1%-40% B; 50-51 min, 40%-100% B; 51-61 min, 100% B; 61-62 min, 100%-1% B, 62-75 min, 1% B. 600 µL of the extract from trypsin digest was injected in two runs (300 µL for each), and the LC-fractions during 26~27 min (T12), 40.5~41.5 min (T8), 43~44 min (T2), 47.5~48.5 min (T6), and 48.5~49.3 min (T4) were collected and dried at 20 mbar. 2 × 100 µL of 80% acetonitrile/water was added and then dried after vortex.

		Step 2 ('pH 8 RP-LC clean-up'): The fractions containing T12, T2, T8, and T4 were merged into one vial and then subjected to pH 8 RP-LC cleanup. The fraction containing T6 was collected with 500 µL of pH 8 buffer solution (0.05% formic acid, titrated to pH 8.0±0.1 with NH_3 (25%, aq)), then 1.54 mg of dithiothreitol was added (37°C, 1 hour), and finally subjected to pH 8 RP-LC cleanup. The column used was Jupiter C4, 300 Å, 5 µm, 250 × 10 mm. Mobile phase A: water (0.05% HCOOH (v/v), titrated to pH 8.0±0.1 with NH_3 (25%, aq)); mobile phase B: 80% (v/v) acetonitrile/water (0.05% HCOOH (v/v), titrated to pH 8.0±0.1 with NH_3 (25%, aq)). Flow rate and column temperature were set the same as in Step 1. The elution gradient was as follows: 0-5 min, 0% B; 5-60 min, 0%-44% B; 60-61 min, 44%-99% B; 61-68 min, 90% B; 68-69 min, 90%-0% B; 69-82 min, 0% B. The dried fractions from Step 1 were redissolved in 500 µL of mobile phase A and injected in two runs (250 µL for each). The LC fractions collected were: 20-21 min (T12), 43-44 min (T8), 51-52 min (T4), and 54-55 min (T2). 100 µL of HCOOH solution (5%, v/v) was added and then dried at 20 mbar. 5. Preparation for LC-MS/MS analysis. 100 µL of 5% HCOOH was added to each collected vial and then evaporated to dryness. The fractions containing T2, T4, T8, and T12 were directly submitted to LC-MS/MS analysis. For the cysteine-containing peptide T6, reduction of disulfide links and carbamidomethylation were performed to convert the entire amount of peptide into a single form prior to MS. The fraction with T6 was redissolved in 80 µL of NH_4HCO_3 solution (50 mM, pH 8, containing 0.8 mg of dithiothreitol) and kept at 37 °C for 1 hour with gentle shaking. Then, 50 µL of NH_4HCO_3 solution (50 mM, pH 8, containing 4 mg of iodoacetamide) was added and kept in the dark for 1 hour at 25 °C. Finally, the solutions were subjected to LC-MS/MS analysis. Samples were analyzed on a Vanquish UHPLC-TSQ Altis MS instrument (Thermo Fisher, USA) operated in LC-MS/MS electrospray ionization (ESI+) mode.
HSA	T2, T4, T6, T8, T12, T13 and T20	Four calibration blends (CBs) were prepared by gravimetrically mixing the 250 mg of calibration standard solution (22kDa GH) and isotope labeled internal standard solution (U-^{15}N 22 kDa GH) to obtain the isotope mass ratios close to 0.5, 0.8, 1.2, and 2.0, respectively. The sample blends (SBs) were prepared by gravimetrically spiking 30 mg of isotope labeled internal standard (U-^{15}N 22 kDa GH) into 250 mg of sample solution (CCQM-K177). The isotope mass ratio in the sample blends was controlled to be within the range of the calibration curve. 250 µL of sample blends (SBs) and calibration blends (CBs) were subjected to acetonitrile precipitation by adding a mixture of 500 µL of water and 1125 µL of acetonitrile, followed by sonication in a water bath for 10 mins. The supernatants retrieved from the CBs and SBs after centrifugation (14,000 rpm for 20 mins at 18 °C) were dried under nitrogen at 30 °C to less than 1mL, and then freeze-dried overnight. The dried CBs and SBs powder were reconstituted with 80 µL of water, NuPAGE™ lithium dodecyl sulfate sample loading buffer (ThermoFisher Scientific) and Bolt™ sample reducing agent (ThermoFisher Scientific) each, and boiled at 95 °C for 5 mins. 38 µL of each CB and SB were loaded into SDS-PAGE gels. Upon completion of SDS-PAGE, the gels were stained with Coomassie blue solution and in-gel digestion was performed using 2 µg of trypsin for each excised gel bands. The extracted peptide solutions were dried under nitrogen at 30 °C prior to reconstitution with 100 µL of 2 % acetonitrile and 1 % formic acid in water, and injected into LC-MS/MS system for quantitation by MRM-MS. Instrument: SCIEX Triple Quad 6500+ Mass Spectrometry coupled with Agilent 1290 Infinity II HPLC

NMIJ	T12	Sample pre-treatment was similar to the procedure used in laboratory of PTB. T12-containing fractions were submitted to LC-MS analysis. LC-MS instrumentation: Nexera high-pressure gradient system (Shimadzu), coupled to a maXis II mass spectrometer (Bruker).
PTB	T12 and T6	Calibrator was prepared by spiking blank serum with GH reference material. Sample and calibrator were spiked with equal amounts of U-^{15}N-GH. Whole serum tryptic digestion of sample and calibrator was followed by two-step clean-up of T6 and T12 using semi-preparative HPLC with eluents at low pH (step 1) and high pH (step 2). For analysis of T6- or T12-containing extracts, a LTQ Orbitrap Elite hybrid mass spectrometer (Thermo Scientific) was used in combination with an Agilent 1200 series LC system, a HESI-II probe and a FAIMS-interface.
GLHK	T12	Sample (250 mg) was spiked with U-^{15}N-GH, which was used as internal standard. Calibration mixtures containing GH (reference material) and U-^{15}N-GH in blank serum were prepared. Whole serum tryptic digestion of samples and calibration mixtures was followed by two-step clean-up of T12 using semi-preparative HPLC with eluents at low pH (step 1) and high pH (step 2). T12-extracts were analysed using LC-MS/MS. LC-MS/MS System: (Agilent 1290 Infinity II Series UHPLC coupled with SCIEX QTRAP 6500+)

Discussion and Conclusions

One study participant and the IFCC laboratory purified intact GH before tryptic digestion, with the participant using gel electrophoresis following precipitation of high-abundant proteins, and the IFCC laboratory using immunoprecipitation. The others purified signature peptides of GH following whole serum digestion. The variability, evaluated as coefficient of variation (CV) of reported results between participants of this key study, was 22.5 % for total-GH. The variability was higher than observed for the study materials of the foregoing pilot study CCQM-P164. For the two spiked blank serum materials of the pilot study the variability between participants was 10% and 13%. If compared to the variability between the four immunoassays (CV=11%), the mass spectrometry-based methods would not be fit for purpose of providing reference values at the present time. In a previous clinical study, a median CV of 24% was observed between eight routine GH-immunoassays, which were used for the measurement of GH in 118 patient sera at concentrations between 5 and 10 ng/mL (Müller et al., Clin Chem Lab Med, 2011, 49, 1135–1142). More recently, the variability between 5 different routine GH-immunoassays was found to be 21% (CV) at GH levels in the range of 0-20 ng/mL in 100 patient sera. (Katsumata N. et al., Endocrine Journal, 2016, 63, 933-936). These findings, together with the results of this key comparison, indicate that significant improvements are still needed for IDMS methods to serve as an SI-traceable reference for routine testing. Various statistical methods were evaluated for defining the Key Comparison Reference Value (KCRV). The combined uncertainty of the DerSimonian and Laird value and the individual uncertainties reported by participants appears to be an appropiate estimation of the overall uncertainty. The excess of inter-laboratory variance (τ) was evaluated with the NIST Consensus Builder for generating key comparison reference values (NICOB). Despite using aliquots of the same reference material and the same stock solution of U-^{15}N-labeled 22 kDa GH, there was considerable variability between participating laboratories. The use of the intact protein in native and labeled form enabled participants to adjust for eventually occuring issues arising from incomplete digestion of GH. One source of measurement uncertainty that has yet to be addressed is the equilibration step after spiking the labeled 22 kDa-GH into the sample. The unknown sources of measurement uncertainty, indicated by the "dark uncertainty" in this study, should be identifiable through refined experimental design, and the required improvements appear to be within the capabilities of the participating laboratories.

Participants whose reported uncertainties overlapped with the DerSimonian & Laird KCRV and were comparable to the uncertainty of the KCRV can claim a Calibration and Measurement Capability (CMC) based on their laboratory's results. Others have to use the standard uncertainties including dark uncertainty, which are given in Table 7. However, combining this dark uncertainty with individual laboratory uncertainties likely results in an overestimation of individual laboratory uncertainties. This seems particularly plausible when one considers the impact of the very different sample preparation methods on the variability of the results.

Beyond the demand for the determination of only total-GH in the present study, three NMIs and one participating IFCC-laboratory demonstrated a capability of isoform-specific GH measurement. This was realized by quantification of either T4 or T6 peptides, as both peptides specifically probe for 22 kDa-GH, while excluding the 20 kDa form.

Core Competencies and How Far Does the Light Shine Statement

The comparison enabled participating NMIs/DIs to demonstrate their measurement capabilities in the determination of a low-abundant protein in the range of 0.23-0.90 pmol/g in human serum.

Acknowledgements

The Coordinators of this comparison would like to express their gratitude to all the participating institutes for their contributions.

Appendixes

Technical protocol

Study Protocol

CCQM-K177

Human Growth Hormone in serum: The determination of the mass fraction of total human growth hormone in serum

Key comparison

Track A

Study protocol

December 2021

Cristian Arsene, Anne-Katrin Römmert, André Henrion

Physikalisch Technische Bundesanstalt (PTB)

Braunschweig, Germany

Background

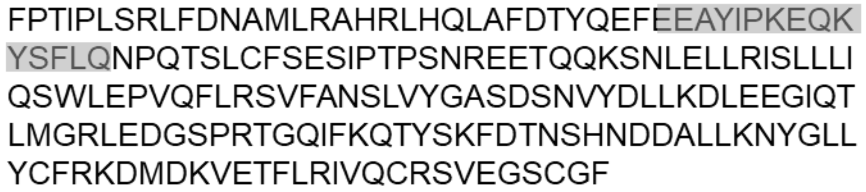
During the PAWG - video conference in April 2021, the group decided to proceed with a key Track A study on the determination of growth hormone (GH) in a pooled human blood serum sample. The small protein, containing 191 amino acids and smaller isoforms, serve as a model for the determination of low-abundant proteins in serum.

Objectives

This comparison enables the participating NMIs/DIs to demonstrate their measurement capabilities in quantification of a protein located in section D of the matrix model of the PAWG with a molar mass ≤ 100 kDa in a biological fluid at a concentration range around 0.50 pmol/g (0.23-0.90 pmol/g).

Measurand

The measurand for the comparison is total-GH in serum at physiological levels. Optionally, the capability of isoform-differential determination of GH can be demonstrated by quantifying 22 kDa-GH or other single isoforms as well as total-GH. The isoforms can be differentiated based on small differences in their primary structure. The difference between the 20 kDa- and 22 kDa-GH is shown in Figure 1.



FPTIPLSRLFDNAMLRAHRLHQLAFDTYQEFEEAYIPKEQK
YSFLQNPQTSLCFSESIPTPSNREETQQKSNLELLRISLLLI
QSWLEPVQFLRSVFANSLVYGASDSNVYDLLKDLEEGIQT
LMGRLEDGSPRTGQIFKQTYSKFDTNSHNDDALLKNYGLL
YCFRKDMDKVETFLRIVQCRSVEGSCGF

Figure 1: Primary structure of 22 kDa-GH. The sequence stretch highlighted in grey is absent in 20 kDa-GH.

Material for comparison

The material prepared for this Key comparison is a frozen pooled human serum. The serum pool was prepared in the laboratory of Physikalisch-Technische Bundesanstalt (PTB) from blood samples of male and female donors. The samples were obtained from a blood bank (DRK-Blutspendedienst NSTOB, Springe, Germany) and from Laboratorium Klin. Chemie & Haematologie University Medical Center Utrecht, Netherlands. The quantity of GH in the final serum pool was tested using an *electrochemiluminescence* immunoassay (ECLIA) in a routine laboratory (LADR, Geesthacht, Germany) and found to match the prerequisite for the preparation of a material containing GH in the range of 5-20 ng/g. The material was re-packed in plastic tubes containing 3 mL aliquots of serum each.

The homogeneity of the study material was evaluated using the immunoassay. Twelve bottles were randomly selected and three subsamples were taken from each bottle. The material was found to be sufficiently homogeneous. At 95% level of confidence, the relative standard uncertainty of inhomogeneity was found to be 0.65 %.

The stability of material was tested using the immunoassay and an isochronous design. The material was found to be stable at -20°C within the proposed timescale for the comparison. The material was also found to be stable at 5°C for 2 weeks and at 25°C for 48 hours, proving the stability during transportation of materials to participating laboratories and during sample preparations for analysis.

Registration

For participation, please contact the study coordinator by e-mail and provide contact details and other necessary information for the shipment of materials.

Instructions for participants

Each participant will receive a vial containing a 3mL-aliquot of the material for comparison. Additionally, an aliquot of serum containing a defined amount of the 22 kDa-GH reference material (WHO IS 98/574, NIBSC, UK) will be available for the preparation of calibration mixtures. The reference material was value-assigned in the study-coordinating laboratory using amino acid analysis based on isotope dilution mass spectrometry (ID-MS). This material was also value-assigned in the laboratory of LGC Group (UK) using peptide-based ID-MS (Pritchard, C. et al. Clin Chem, 2009, 55, 1984-1990).

Next to an aliquot of serum containing the reference material, an aliquot of serum containing the labeled 22 kDa GH (U-^{15}N 22 kDa GH) will be sent to participants. Blank serum will also be available for the preparation of calibration mixtures. All vials will be shipped by airmail under cooling conditions (5 °C to -20 °C) using thermo-boxes and ice packs.

Please note: The provider of blood samples has tested all samples negatively for infectious agents as reported in the Appendix. Despite this, the materials should be handled as biohazardous and as if capable of transmitting infectious diseases.

After receipt, all vials should be immediately transferred to low temperature storage (≤ -20 °C). For analysis, the vials should be equilibrated to ambient temperature (20 °C to 25 °C) for 1 hour until materials are thawed. All materials should be homogenized by gentle swirling before sampling. For analysis, the recommended minimum amount of sample or calibration mixture is 250 mg. For calibration, all participants should use the reference material and the material containing the labeled 22 kDa GH provided by the study coordinator. The stock solutions of reference material and labeled 22 kDa GH should be diluted with provided blank serum to obtain solutions at appropriate GH-concentrations for standard additions. Participants may use their own methods for the determination of signature peptides of GH after tryptic digestion of the sample and the calibration mixture. For isoform-differential determination it is recommended to determine one signature peptide of 22 kDa-GH i.e. T4 (LHQLAFDTYQEFEEAYIPK) or T6 (YSFLQNPQTSLCFSESIPTPSNR) and another one of total-GH e.g. T12 (LEDGSPR) or even more to underpin the capability of accurate determination using different signature peptides.

Reporting of results

Participating NMIs/DIs will be provided with a form for reporting results. They are expected to report their results based on at least 3 subsamples of the study material. Results should be reported as mass fractions (in ng/g) of GH (22 kDa GH and total-GH, or only total-GH) in the material. For this key comparison, a reference value (KCRV) will be calculated only for total-GH. The molar mass of 22 kDa-GH (22124.8 g/mol) should be used for calculating the mass fraction of 22kDa-GH and total-GH. Reported results should include standard and expanded uncertainties at the 95 % level of confidence for single results determined from the replicate determinations. Information about the measurement procedure and the calculation of results including the estimation of measurement uncertainty should be given. Completed forms with final results should be sent to Anne-Katrin Römmert, a member of the coordinating laboratory (PTB), before the scheduled deadline.

Core Competency

For the first time in the protein analysis group (PAWG), participating NMIs/DIs are able to demonstrate their capability to determine a serum protein i.e. GH as the entire set of its isoforms (total-GH) in the physiological concentration range around 0.50 pmol/g. Optionally participants can demonstrate their capability to determine fractions of GH isoforms next to total-GH.

Schedule

Call for participation: December 2021

Deadline for registration: January 2022

Sample distribution: January/February 2022

Deadline for submission of results: September 2022

Coordinating Laboratory

Physikalisch-Technische Bundesanstalt (PTB)
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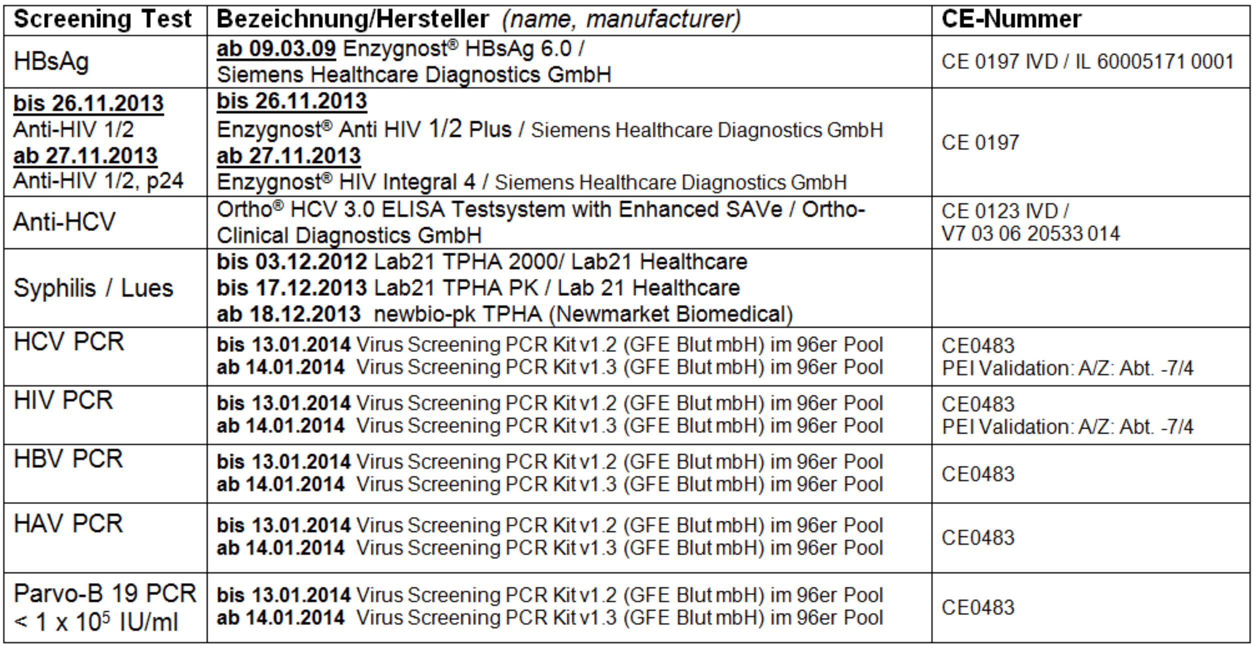
Appendix

All blood samples used for the preparation of comparison materials were tested for infectious agents and found to be negative with the following test-kits:



Screening Test	**Bezeichnung/Hersteller** *(name, manufacturer)*	**CE-Nummer**
HBsAg	**ab 09.03.09** Enzygnost® HBsAg 6.0 / Siemens Healthcare Diagnostics GmbH	CE 0197 IVD / IL 60005171 0001
bis 26.11.2013 Anti-HIV 1/2 **ab 27.11.2013** Anti-HIV 1/2, p24	**bis 26.11.2013** Enzygnost® Anti HIV 1/2 Plus / Siemens Healthcare Diagnostics GmbH **ab 27.11.2013** Enzygnost® HIV Integral 4 / Siemens Healthcare Diagnostics GmbH	CE 0197
Anti-HCV	Ortho® HCV 3.0 ELISA Testsystem with Enhanced SAVe / Ortho-Clinical Diagnostics GmbH	CE 0123 IVD / V7 03 06 20533 014
Syphilis / Lues	**bis 03.12.2012** Lab21 TPHA 2000/ Lab21 Healthcare **bis 17.12.2013** Lab21 TPHA PK / Lab 21 Healthcare **ab 18.12.2013** newbio-pk TPHA (Newmarket Biomedical)	
HCV PCR	**bis 13.01.2014** Virus Screening PCR Kit v1.2 (GFE Blut mbH) im 96er Pool **ab 14.01.2014** Virus Screening PCR Kit v1.3 (GFE Blut mbH) im 96er Pool	CE0483 PEI Validation: A/Z: Abt. -7/4
HIV PCR	**bis 13.01.2014** Virus Screening PCR Kit v1.2 (GFE Blut mbH) im 96er Pool **ab 14.01.2014** Virus Screening PCR Kit v1.3 (GFE Blut mbH) im 96er Pool	CE0483 PEI Validation: A/Z: Abt. -7/4
HBV PCR	**bis 13.01.2014** Virus Screening PCR Kit v1.2 (GFE Blut mbH) im 96er Pool **ab 14.01.2014** Virus Screening PCR Kit v1.3 (GFE Blut mbH) im 96er Pool	CE0483
HAV PCR	**bis 13.01.2014** Virus Screening PCR Kit v1.2 (GFE Blut mbH) im 96er Pool **ab 14.01.2014** Virus Screening PCR Kit v1.3 (GFE Blut mbH) im 96er Pool	CE0483
Parvo-B 19 PCR $< 1 \times 10^5$ IU/ml	**bis 13.01.2014** Virus Screening PCR Kit v1.2 (GFE Blut mbH) im 96er Pool **ab 14.01.2014** Virus Screening PCR Kit v1.3 (GFE Blut mbH) im 96er Pool	CE0483

Participants' Reports

Report of results

CCQM-K177

"Human Growth Hormone in serum: The determination of the mass fraction of total human growth hormone in serum"

Participating Laboratory

Institute: National Institute of Metrology, China

Reporting date: September 19, 2022

Contact person: Rui Zhai，Zhanying Chu，Youxun Jin，Liqing Wu

E-mail: zhairui@nim.ac.cn

Overall Results

Mass fraction of total-GH[1] in serum (mean of single results from replicate determinations in ng/g)	Combined Standard Uncertainty (ng/g)	Coverage Factor (*k*)	Expanded Uncertainty (ng/g)
15.02	**0.98**	**2**	**1.96**

optional:

Mass fraction of 22kDa-GH[1] in serum (mean of single results from replicate determinations in ng/g)	Combined Standard Uncertainty (ng/g)	Coverage Factor (*k*)	Expanded Uncertainty (ng/g)
14.26	**0.67**	**2**	**1.34**

[1]The molar mass of 22kDa-GH is 22124.8 g/mol, also to be used with total-GH measurements.

Single results

Signature peptide (e.g. T12…)	Mass fraction of total-GH in aliquot 1 of sample (ng/g)	Mass fraction of total-GH in aliquot 2 of sample (ng/g)	Mass fraction of total-GH in aliquot 3 of sample (ng/g)	Mass fraction of total-GH in aliquot 4 of sample (ng/g)
T2	**15.93**	**16.29**	**16.31**	**16.99**
T8	**14.39**	**14.93**	**13.87**	**14.68**
T12	**13.37**	**14.07**	**14.81**	**14.56**
Signature peptide (e.g. T4, T6)	Mass fraction of 22kDa-GH in aliquot 1 of sample (ng/g)	Mass fraction of 22kDa-GH in aliquot 2 of sample (ng/g)	Mass fraction of 22kDa-GH in aliquot 3 of sample (ng/g)	Mass fraction of 22kDa-GH in aliquot 4 of sample (ng/g)
T4	**14.65**	**14.82**	**13.14**	**13.73**
T6	**/**	**14.46**	**14.55**	**14.49**

Analytical conditions

Sample treatment

Sample amount used for analysis (mg)	About 500 mg per analysis.

Brief description of the measurement (e.g. preparation of sample and calibrator, digestion, method and instrumentation used for purification of the analyte)	**1. Standard Preparation.** A stock standard of the GH (297.17 fmol/mg, 6.57 ng/mg) and L-GH (292.14 fmol/mg, 6.54 ng/mg) in serum were diluted 22 fold gravimetrically with blank serum. The diluted serum was mixed gently for 1 h before the preparation of calibrators and samples. **2. Sample and Calibrator Preparation.** 500 μL of serum was taken and mixed with the L-GH. The calibrator contained 500 μL of blank serum with the GH (about 14.0 ng/g) standard and L-GH (about 14.0 ng/g). The amount of the labeled protein was equivalent to GH in both calibrators and samples. **3. Tryptic Digestion** The serums were incubated at 37 °C while gently shaking. 65 μL of TRIS-solution (302.5 mg of TRIS (base) and 395 mg of TRIS•HCl dissolved in 1 mL of water) was added into 500 μL serum (samples or calibrators, resp.). 50 μL of trypsin solution (12 mg of trypsin dissolved in 1.8 mL of 50 mM HAc) was added dropwise. Then 100 μL of acetonitrile was added after every 30 min dropwise for 11 times, and 50 μL of trypsin solution was added after 30, 90, 150, 210 and 270 min. And the incubation was continued over night. 3.2 mg of dithiothreitol was added and the calibrators and samples were gently shaken at 37 °C for 1 h. The precipitate was spun down for 2 × 10 min at 17860 g. After the first centrifugation, the supernatant was separated from the precipitate and the remaining pellet was washed with 1 mL of water/ acetonitrile (50/50, v/v). After vigorous vortex and ultrasonic treatment for 1 min, vortex again. After the second centrifugation, the 'wash solution' was added to the liquid phase from the first centrifugation. All the collected liquid was dried at 20 °C under 20 mbar. The dried residue was extracted with 400 μL of water. After vortex, 100 μL of TFA (16%) was added, vortex again. 10 min of centrifugation at 3960 g was performed and the supernatant was separated. The remaining pellet was washed with 300 μL of water. After vertex and the second centrifugation at 3960 g for 10 min, the supernatant was added into the liquid phase obtained in the previous step. **4. Sample Cleanup.** Two fold liquid chromatography was employed for extraction of targeted signature peptides after serum trypsin digest. **Step 1 ('pH 2 RP-LC clean-up'):** The column Jupiter Proteo (RP-C12, 90Å, 4 µm, 250 × 10 mm) was used. Mobile phase A: water (0.1% CF_3COOH, v/v); mobile phase B: acetonitrile (0.1% CF_3COOH, v/v). The flowrate was set at 2 mL/min

and the chromatographic separation was performed at 25 ^{o}C. The elution gradient was as follows: 0-5 min, 1% B; 5-50 min, 1%-40% B; 50-51 min, 40%-100% B; 51-61 min, 100% B; 61-62 min, 100%-1% B, 62-75 min, 1% B. 600 µL of the extract from trypsin digest was injected in two runs (300 µL for each), and the LC-fractions during 26~27 min (T12), 40.5~41.5 min (T8), 43~44 min (T2), 47.5~48.5 min (T6), and 48.3~49.3 min (T4) were collected and dried at 20 mbar. 2 × 100 µL of 80% acetonitrile/water was added and then dried after vortex.

Step 2 ('pH 8 RP-LC clean-up'):

The fraction containing T12, T2, T8, T4 were merged into one vial and then performed pH 8 RP-LC clean up. The fraction containing T6 was collected with 500 µL pH 8 buffer solution (0.05% formic acid, titrated to pH 8.0±0.1 with NH_3(25%, aq)), then, 1.54 mg dithiothreitol was added (37°C, 1h), and finally performed pH 8 RP-LC clean up.

The column Jupiter C4, 300 Å, 5 µm, 250 × 10 mm was used. Mobile phase A: water (0.05% HCOOH (v/v), titrated to pH 8.0±0.1 with NH_3(25%, aq)); mobile phase B: 80% (v/v) acetonitrile/water (0.05% HCOOH (v/v), titrated to pH 8.0±0.1 with NH_3(25%, aq)). Flowrate and column temperature were set as same as Step 1. The elution gradient was as follows: 0-5 min, 0% B; 5-60 min, 0%-44% B; 60-61 min, 44%-99% B; 61-68 min, 90% B; 68-69 min, 90%-0% B; 69-82 min, 0% B. The dried fractions from Step 1 was redissolved in 500 µL of mobile phase A and was injected in two runs (250 µL for each), and the LC-fractions during 20~21 min (T12), 43~44 min (T8), 51~52 min (T4) and 54~55 min (T2) were collected. 100 µL of HCOOH solution (5%, v/v) was added and then dried at 20 mbar.

5. Preparation for LC-MS/MS analysis.

Add 100 µL of 5% HCOOH into each collected vial and then evaporate to dryness. The fractions with T2, T4, T8 and T12 were directly proceeded to LC-MS/MS analysis.

For that T6 is the cysteine-containing peptide, reduction of disulfide links and carbamido-methylation was performed so as to transfer the whole amount peptide into just one form prior to MS. The fraction with T6 was redissolved with 80 µL of NH_4HCO_3 solution (50 mM, pH8, containing 0.8 mg of dithiothreitol) and kept at 37 ^{o}C for 1 h with gently shaking. Then 50 µL of NH_4HCO_3 solution (50 mM, pH8, containing 4 mg of iodoacetamide) was added and kept in dark for 1 h at 25 ^{o}C. Finally, the solutions were proceeded to LC-MS/MS analysis.

Instrumentation and MS-acquisition

Instrumentation used (e.g., type of liquid-chromatograph and mass spectrometer, manufacturer, important instrument parameters, analytical technique used: LC-MS or LC-MS/MS etc.	Samples were analyzed on a Vanquish UHPLC-TSQ Altis MS instrument (Thermo Fisher, USA) operated in LC-MS/MS electrospray ionization (ESI+) mode.
Indicate signature peptides of total-GH/22kDa-GH and MS-acquisition parameters (ion/MRM, collision energy)	The mass spectrometer was run in a multiple reaction monitoring (MRM) mode with quadrupole mass filter Q1 and Q3 at unit resolution. Peptides (T2, T8 and T12) were selected for the determination of total-GH, and peptides (T4 and T6) were selected for the determination of 22-kDa GH. The monitored transitions were as follow: m/z 490.30→719.32 and m/z 496.28→729.29 for **T2** and **T2*** ($[M+2H]^{2+}\rightarrow y6$), collision energy (CE) = 16.79 V; m/z 422.75→401.17 and m/z 428.18→407.15 for **T8** and **T8*** ($[M+2H]^{2+}\rightarrow y3$), CE = 15.57 V; m/z 387.19→531.24 and m/z 392.18→539.22 for **T12** and **T12*** ($[M+2H]^{2+}\rightarrow y5$), CE = 13.75 V; m/z 781.58→1050.21 and m/z 789.56→1060.68 for **T4** and **T4*** ($[M+3H]^{3+}\rightarrow b17^{2+}$), CE = 15.28 V; m/z 891.92→671.36 and m/z 901.89→681.33 for **T6** and **T6*** ($[M+3H]^{3+}\rightarrow y6$), CE = 20.75 V (sulfydryl in T6 was reacted with iodoacetamide); Fragmentation was induced using nitrogen at a high-pressure setting. The ESI source was operated at a 3500 V sprayer voltage and a 315 °C dry gas temperature. All other parameters were optimized to obtain maximum signal intensities for each peptide.
Calibration method/design used	Single point calibration mode was used for both sample measurement.

Chromatographic Column (i.e., type and manufacturer)	Aeris Peptide XB-C18 3.6 μm column, 2.1 × 250 mm (Phenomenex).
Chromatographic Conditions (eluents, gradient and flow rate)	Mobile phases were (A) water/0.1% formic acid (v/v) and (B) acetonitrile/0.1% formic acid (v/v). Linear gradient: 0-6 min, 98% A, 6-15 min, 98%-91% A, and 15-70 min, 91%-74.5% A, followed by washing at 0% A and re-equilibration. The flow rate was 300 μL/min. Injection volume was 40 μL. Column tempeature was 45 ℃. Under these conditions, the retention time of T12, T8, T2, T6 and T4 was 10.5 min, 28.7 min, 37.2 min, 60.6 min and 60.8 min, respectively.
Measurement equation used to determine the mass fraction of total-GH/22kDa-GH in serum	The total-hGH/22-kDa-GH mass fraction in serum was calculated by the following formula: $c8=\frac{m5m1c1A2m9}{m8(m1+m2)(m5+m6+m7)A1}$ **Where:** $m1$: Weight of original hGH standard solution. $m2$: Weight of blank serum added to the preparation of hGH stock solution. $m5$: Weight of the hGH working solution added to the preparation of calibrator solution. $m6$: Weight of the L-hGH working solution added to the preparation of calibrator solution. $m7$: Weight of blank serum added to the preparation of calibrator solution. $m8$: Weight of the sample. $m9$: Weight of the L-hGH in the spiked sample. $c1$: Concentration of the original hGH standard solution. $c8$: Concentration of the sample. $A1$: Peak area ratio of the signature peptide to its isotopic one in the calibrator.

	*A*2: Peak area ratio of the signature peptide to its isotopic one in the sample.
Uncertainty budget (please list all components and their contribution to the combined standard uncertainty)	**1. Uncertainty budget for total-GH measeurment** The measurement model was following: $$c8=\frac{m5m1c1A2m9}{m8(m1+m2)(m5+m6+m7)A1}$$ The following uncertainty sources were taken into consideration: **1.1** Balance and GH standard： Each weighing was performed on a ME235S balance. The imprecision of the balance was used to calculate the uncertainty of each weighing with a rectangle distribution. The imprecision was 0.01 mg. Therefore, the uncertainty component of each weighing was $$u_1=\frac{0.00001}{\sqrt{3}}=5.77\times10^{-6}\mathrm{g}$$ The relative expanded uncertainty of the GH standard was 2.2% with a coverage factor of 2, which was provided by PTB. Based on the measurement model, the sensitivity factor of each weighing and GH standard was calculated. $$c_i=\frac{\partial f}{\partial x_i}$$ Next, all the uncertainty components were combined to get the type B uncertainty, which was shown in Table 1. $$u_b=\sqrt{\sum_{i=1}^{n}\left[\frac{\partial f}{\partial x_i}u_i\right]^2}$$ **Table 1**. Uncertainty budget of sample 1 **(Type B)**

Uncertainty component	Value	Sensitivity factor	Uncertainty	Uncertainty component
m1	2.99×10^{-2}	4.41×10^{-7}	5.77×10^{-7}	2.55×10^{-12}
c1	6.58×10^{-6}	9.48×10^{-5}	7.23×10^{-7}	6.85×10^{-12}
m2	6.46×10^{-1}	-9.22×10^{-10}	5.77×10^{-6}	-5.32×10^{-15}
c3	6.54×10^{-6}	0	N/A	0
m3	7.42×10^{-2}	0	5.77×10^{-6}	0
m4	1.60	0	5.77×10^{-6}	0
m5	2.35×10^{-2}	2.53×10^{-8}	5.77×10^{-6}	1.46×10^{-13}
m6	2.38×10^{-2}	-1.18×10^{-9}	5.77×10^{-6}	-6.82×10^{-15}
m7	4.80×10^{-1}	-1.18×10^{-9}	5.77×10^{-6}	-6.82×10^{-15}
m8	5.02×10^{-1}	-1.24×10^{-9}	5.77×10^{-6}	-7.16×10^{-15}
m9	2.31×10^{-2}	2.70×10^{-8}	5.77×10^{-6}	1.56×10^{-13}
A1	1.06	-5.57×10^{-10}	4.42×10^{-2}	-2.46×10^{-11}
A2	1.19	5.33×10^{-10}	7.17×10^{-2}	3.83×10^{-11}

Therefore, the combined type B uncertainty is:

$$u_{c,\,b}=\sqrt{\sum_{i=1}^{9}(c_{mi}u_{mi})^2+(c_{c1}u_{c1})^2+(c_{c3}u_{c3})^2+(c_{A1}u_{A1})^2+(c_{A2}u_{A2})^2\times}$$

$$= 0.05 \text{ ng/g}$$

1.2 Trypsin digestion efficiency

Based upon the experimental data and past experience, in serum materials, the uncertainty component brought by the trypsin digestion efficiency for each peptide (T2, T8 and T12) was estimated to be 3%.

$$u_{Trypsin(\mathrm{rel})} = \sqrt{0.03^2+0.03^2+0.03^2} = 5.2\%$$

$$u_{Trypsin} = \bar{x} \times 5.2\% = 15.02\times0.052 = 0.78\ \mathrm{ng/g}$$

1.3 Method reproducibility:

Type A uncertainty evaluation was used to calculate this uncertainty component, which was calculated based on 4 measurements for total-GH.

Table 2. Quantification results of total-GH with T2, T8 and T12 peptides

Signature peptide	Mass fraction of total-GH (ng/g) (1st analysis)	Mass fraction of total-GH (ng/g) (2nd analysis)	Mass fraction of total-GH (ng/g) (3rd analysis)	Mass fraction of total-GH (ng/g) (4th analysis)
T2	15.93	16.29	16.31	16.99
T8	14.39	14.93	13.87	14.68
T12	13.37	14.07	14.81	14.56

The standard deviation was estimated by range method:

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

$$u_A = s(\bar{x}) = s(x)/\sqrt{4} = 0.58\ \mathrm{ng/g}$$

The combined standard uncertainty is:

$$u_{c,\ \mathrm{total\text{-}GH}}=\sqrt{u_A^2+u_{c,\ b}^2+u_{Trypsin}{}^2} = \sqrt{0.58^2+0.05^2+0.78^2}$$

$$= 0.98\ \mathrm{ng/g}$$

$$u_{c\ (\text{total-GH},\ rel)}=\frac{u_{c,\ \text{total-GH}}}{\bar{x}}\times 100\%=6.49\%$$

Therefore, the expanded relative uncertainty is:

$$U_{exp\ (rel)} = k\cdot u_{c\ (\text{total-GH},\ rel)} = 2 \times 6.49\% = 13.0\%$$

Therefore, the result can be expressed as (15.02 ± 1.96) ng/g.

2. Uncertainty budget for 22 kDa-GH measeurment

The measurement model was following:

$$c8=\frac{m5m1c1A2m9}{m8(m1+m2)(m5+m6+m7)A1}$$

The following uncertainty sources were taken into consideration:

2.1 Balance and GH standard：

Each weighing was performed on a ME235S balance. The imprecision of the balance was used to calculate the uncertainty of each weighing with a rectangle distribution. The imprecision was 0.01 mg. Therefore, the uncertainty component of each weighing was

$$u_1=\frac{0.00001}{\sqrt{3}}=5.77\times 10^{-6}\mathrm{g}$$

The relative expanded uncertainty of the GH standard was 2.2% with a coverage factor of 2, which was provided by PTB.

Based on the measurement model, the sensitivity factor of each weighing and GH standard was calculated.

$$c_i=\frac{\partial f}{\partial x_i}$$

Next, all the uncertainty components were combined to get the type B uncertainty, which was shown in Table 2.

$$u_b=\sqrt{\sum_{i=1}^{n}\left[\frac{\partial f}{\partial x_i}u_i\right]^2}$$

Table 3. Uncertainty budget of sample 1 **(Type B)**

Uncertainty component	Value	Sensitivity factor	Uncertainty	Uncertainty component
m1	2.99×10^{-2}	4.41×10^{-7}	5.77×10^{-7}	2.55×10^{-12}
c1	6.58×10^{-6}	9.48×10^{-5}	7.23×10^{-7}	6.85×10^{-12}
m2	6.46×10^{-1}	-9.22×10^{-10}	5.77×10^{-6}	-5.32×10^{-15}
c3	6.54×10^{-6}	0	N/A	0
m3	7.42×10^{-2}	0	5.77×10^{-6}	0
m4	1.60	0	5.77×10^{-6}	0
m5	2.35×10^{-2}	2.53×10^{-8}	5.77×10^{-6}	1.46×10^{-13}
m6	2.38×10^{-2}	-1.18×10^{-9}	5.77×10^{-6}	-6.82×10^{-15}
m7	4.80×10^{-1}	-1.18×10^{-9}	5.77×10^{-6}	-6.82×10^{-15}
m8	5.02×10^{-1}	-1.24×10^{-9}	5.77×10^{-6}	-7.16×10^{-15}
m9	2.31×10^{-2}	2.70×10^{-8}	5.77×10^{-6}	1.56×10^{-13}
A1	1.06	-5.57×10^{-10}	4.42×10^{-2}	-2.46×10^{-11}
A2	1.19	5.33×10^{-10}	7.17×10^{-2}	3.83×10^{-11}

Therefore, the combined type B uncertainty is:

$$u_{c,\,b}=\sqrt{\sum_{i=1}^{9}(c_{mi}u_{mi})^2+(c_{c1}u_{c1})^2+(c_{c3}u_{c3})^2+(c_{A1}u_{A1})^2+(c_{A2}u_{A2})^2\times}$$

$$= 0.05\ \text{ng/g}$$

2.2 Trypsin digestion efficiency

Based upon the experimental data and past experience, in serum materials, the uncertainty component brought by the trypsin digestion efficiency for each peptide (T4, T6) was estimated to be 3%.

$$u_{Trypsin(\mathrm{rel})} = \sqrt{0.03^2+0.03^2} = 4.2\%$$

$$u_{IAA} = \bar{x} \times 4.2\% = 14.26 \times 0.042 = 0.60 \text{ ng/g}$$

2.3 Method reproducibility:

Type A uncertainty evaluation method was used to calculate this uncertainty component. The value was calculated based on 4 measurements.

Table 4. Quantification results of 22 kDa-GH with T4 and T6 peptides

Signature peptide	Mass fraction of 22 kDa-GH (ng/g) (1st analysis)	Mass fraction of 22 kDa-GH (ng/g) (2nd analysis)	Mass fraction of 22 kDa-GH (ng/g) (3rd analysis)	Mass fraction of 22 kDa-GH (ng/g) (4th analysis)
T4	14.65	14.82	13.14	13.73
T6	/	14.46	14.55	14.49

The standard deviation was estimated by range method:

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

$$u_A = s(\bar{x}) = s(x)/\sqrt{4} = 0.30 \text{ ng/g}$$

The combined standard uncertainty is:

$$u_{c,\ 22\ \mathrm{kDa\text{-}GH}} = \sqrt{u_A^2+u_{c,\ b}^2+u_{IAA}^2} =$$

$$\sqrt{0.30^2+0.05^2+0.60^2} = 0.67 \text{ ng/g}$$

$$u_{c\ (22\ \mathrm{kDa\text{-}GH},\ rel)} = \frac{u_{c,\ 22\ \mathrm{kDa\text{-}GH}}}{\bar{x}} \times 100\% = 4.63\%$$

Therefore, the expanded relative uncertainty is:

	$U_{exp\ (rel)} = k \cdot u_{c\ (22\ \text{kDa-GH},\ rel)} = 2 \times 4.62\% = 9.26\%$ Therefore, the result can be expressed as (14.26 ± 1.34) ng/g.
Additional Comments or Observations	The received vials were immediately transferred to low tempera-ture storage (-80 °C). The temperature recorder in the packing box showed -0.4 ^{o}C when we received the vials. There was a bit of precipitation in the serum material, so a mild centrifugation procedure (2000 rpm, 2 min) was performed before analysis.

Report of results

CCQM-K177

“Human Growth Hormone in serum: The determination of the mass fraction of total human growth hormone in serum”

Participating Laboratory

Institute: Health Sciences Authority

Reporting date: 30 September 2022

Contact person: Qinde Liu, Hong Liu, Hwee Tong Tan, Tang Lin Teo

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Overall Results

Mass fraction of total-GH[1] in serum (mean of single results from replicate determinations in ng/g)	Combined Standard Uncertainty (ng/g)	Coverage Factor (*k*)	Expanded Uncertainty (ng/g)
14.10	**0.82**	**2.31**	**1.89**

optional:

Mass fraction of 22kDa-GH[1] in serum (mean of single results from replicate determinations in ng/g)	Combined Standard Uncertainty (ng/g)	Coverage Factor (*k*)	Expanded Uncertainty (ng/g)
12.94	**0.77**	**2.36**	**1.83**

[1] The molar mass of 22kDa-GH is 22124.8 g/mol, also to be used with total-GH measurements.

Single results

Signature peptide (e.g. T12…)	Mass fraction of total-GH in aliquot 1 of sample (ng/g)	Mass fraction of total-GH in aliquot 2 of sample (ng/g)	Mass fraction of total-GH in aliquot 3 of sample (ng/g)	Mass fraction of total-GH in aliquot 4 of sample (ng/g)
T2, T8, T12, T13, T20	13.02	15.70	14.31	13.37
Signature peptide (e.g. T4, T6)	Mass fraction of 22kDa-GH in aliquot 1 of sample (ng/g)	Mass fraction of 22kDa-GH in aliquot 2 of sample (ng/g)	Mass fraction of 22kDa-GH in aliquot 3 of sample (ng/g)	Mass fraction of 22kDa-GH in aliquot 3 of sample (ng/g)
T4, T6	11.65	14.24	13.53	12.35

Analytical conditions

Sample treatment

Sample amount used for analysis (mg)	250 mg

Brief description of the measurement (e.g. preparation of sample and calibrator, digestion, method and instrumentation used for purification of the analyte)	Four calibration blends (CBs) were prepared by gravimetrically mixing the 250 mg of calibration standard solution (22kDa GH) and isotope labeled internal standard solution (U-^{15}N 22 kDa GH) to obtain the isotope mass ratios close to 0.5, 0.8, 1.2, and 2.0, respectively. The sample blends (SBs) were prepared by gravimetrically spiking 30 mg of isotope labeled internal standard (U-^{15}N 22 kDa GH) into 250 mg of sample solution (CCQM-K177). The isotope mass ratio in the sample blends was controlled to be within the range of the calibration curve. 250 µL of sample blends (SBs) and calibration blends (CBs) were subjected to acetonitrile precipitation by adding a mixture of 500 µL of water and 1125 µL of acetonitrile, followed by sonication in a water bath for 10 mins. The supernatants retrieved from the CBs and SBs after centrifugation (14,000 rpm for 20 mins at 18 ^{o}C) were dried under nitrogen at 30 ^{o}C to less than 1mL, and then freeze-dried overnight. The dried CBs and SBs powder were reconstituted with 80 µL of water, NuPAGE^{TM} lithium dodecyl sulfate sample loading buffer (ThermoFisher Scientific) and Bolt^{TM} sample reducing agent (ThermoFisher Scientific) each, and boiled at 95 ^{o}C for 5 mins. 38 µL of each CB and SB were loaded into SDS-PAGE gels. Upon completion of SDS-PAGE, the gels were stained with Coomassie blue solution and in-gel digestion was performed using 2 µg of trypsin for each excised gel bands. The extracted peptide solutions were dried under nitrogen at 30 ^{o}C prior to reconstitution with 100 µL of 2 % acetonitrile and 1 % formic acid in water, and injected into LC-MS/MS for quantitation by MRM-MS.

Instrumentation and MS-acquisition

Instrumentation used (e.g., type of liquid-chromatograph and mass spectrometer, manufacturer, important instrument parameters, analytical technique used: LC-MS or LC-MS/MS etc.	LC-MS/MS: Instrument: SCIEX Triple Quad 6500+ Mass Spectrometry coupled with Agilent 1290 Infinity II HPLC MS Parameters: CUR: 30.00 CAD: 8.00 IS: 4500.00 TEM: 450.00 GS1: 45.00 GS2: 40.00 EP 10.00 IQ1 -10.00
Indicate signature peptides of total-GH /22kDa-GH and MS-acquisition parameters (ion/MRM, collision energy)	Signature peptides of total-GH: T2: 490.4/719.4, CE = 24; T2 ISD: 496.3/729.3, CE = 24 T8: 422.7/643.5, CE = 19; T8 ISD: 428.4/651.4, CE = 19 T12: 387.2/531.4, CE = 18; T12 ISD 392.2/539.1, CE = 18 T13: 347.3/592.4, CE = 17; T13 ISD 351.4/599.2, CE = 17 T20: 338.3/463.2, CE = 16; T20 ISD 342.8/470.2, CE = 16 Signature peptides of 22kDa-GH: T4: 781.5/244.2, CE = 30; T4 ISD: 789.5/247.2, CE = 30 T6: 891.8/671.3, CE = 24; T6 ISD: 901.8/681.3, CE = 24

Calibration method/design used	Multi-level (4-points calibration curve) IDMS method was used. Four calibration blends were prepared by gravimetrically mixing the calibration standard solution and the isotope labeled internal standard solution to obtain the isotope mass ratio close to 0.50, 0.80, 1.2, and 2.00, respectively. The sample blend was prepared by gravimetrically spiking isotope labeled internal standard into the sample solution. The isotope mass ratio of the sample blend was controlled to be within the range of the calibration curve.
Chromatographic Column (i.e., type and manufacturer)	Agilent ZORBAX 300SB–C18, 2.1 x 150 mm, 5 μm
Chromatographic Conditions (eluents, gradient and flow rate)	Mobile phase A: 0.1% formic acid in water. Mobile phase B: 0.1% formic acid in acetonitrile. Flow rate: 0.3 mL/min 0 – 2 min, 5% B 2 – 2.5 min, 5% to 20% B 2.5 – 5 min, 20% B 5 - 10 min, 20% to 30% B 10 - 11 min, 30% to 40% B 11 – 11.5 min, 40% to 80% B 11.5 – 12.5 min, 80% B 12.5 – 13 min, 80% to 5% B 13 – 16 min, 5% B 16 min, stop

Measurement equation used to determine the mass fraction of total-GH/22kDa-GH in serum	The mass fractions of total-GH and 22kDa-GH in the sample were calculated based on the IDMS calibration curve as follows: $C_X = (mR_B + b)\times \frac{W_Y}{M_X} = (mR_B + b)\times \frac{M_Y C_Y}{M_X}$ (1) where C_X = mass fraction of total-CH or 22kDa-GH in the sample (ng/g) M_X = mass of sample (determined by weighing) (g) M_Y = mass of isotope labeled standard solution (determined by weighing) (g) W_Y = mass of the isotope labeled standard spiked into the serum sample (equals to $M_Y \times C_Y$) (ng) R_B = peak area ratio of sample blend for different signature peptides (determined by LC-MS/MS measurements) C_Y = concentration of isotope labeled standard solution (determined by weighing and from concentration of the isotope labeled standard) (ng/g) m = gradient of the slope of linear regression plot (determined by the linear fit of the isotope mass ratio from weighing and the peak area ratio from LC-MS/MS measurement of the calibration blends) b = intercept on y axis of the linear regression plot (determined by the linear fit of the isotope mass ratio from weighing and the peak area ratio from LC-MS/MS measurement of the calibration blends)
Uncertainty budget (please list all components and their contribution to the combined standard uncertainty)	See Appendix A below
Additional Comments or Observations	NA

Uncertainty Budget

As C_Y does not contribute to the measurement uncertainty of C_X, for the estimation of uncertainty, considering $R_M = mR_B + b$, and let $R_M = R_M{'}C_Z/C_Y$, Equation (1) is converted to:

$$C_X = R_M{'} \times \frac{M_Y C_Z}{M_X} \tag{2}$$

Where,

R_M = isotope mass ratio in sample blend

C_Z = concentration of 22kDa-GH in the calibration standard solution (ng/g)

A standard uncertainty was estimated for all components of the measurement in Equation (2), which were then combined using respective derived sensitivity coefficients to estimate a combined standard uncertainty in the reported result of total-GH and 22kDa-GH in the study sample. A coverage factor *k* (2.31 for total-GH and 2.36 for 20kDa-GH) associated with the degree of freedom was used to expand the combined standard uncertainty at a 95 % confidence interval. Possible sources of biases [between-subsample variation (F_{SV}), SDS-PAGE separation (F_{SDS}), and between-peptide variation (F_{PV})] were accounted for in the final uncertainty budget with the use of the measurement equation:

$$C_X = F_{SV} \times F_{SDS} \times F_{PV} \times R_M' \times \frac{M_Y C_Z}{M_X} \tag{3}$$

The sensitivity coefficients of each component can be expressed as follows:

$$\frac{\partial C_X}{\partial M_Y} = \frac{C_X}{M_Y} \qquad \frac{\partial C_X}{\partial M_X} = -\frac{C_X}{M_X} \qquad \frac{\partial C_X}{\partial C_Z} = \frac{C_X}{C_Z} \qquad \frac{\partial C_X}{\partial R_M{'}} = \frac{C_X}{R_M{'}}$$

$$\frac{\partial C_X}{\partial F_{SV}} = \frac{C_X}{F_{SV}} \qquad \frac{\partial C_X}{\partial F_{SDS}} = \frac{C_X}{F_{SDS}} \qquad \frac{\partial C_X}{\partial F_{PV}} = \frac{C_X}{F_{PV}}$$

The standard uncertainty of each component was calculated as follows:

(1) M_Y and M_X: Calculated based on the uncertainty of the balance obtained from the calibration report.

(2) C_Z: The uncertainty associated with the concentration of the diluted 22kDa-GH standard solution provided by the coordinator.

(3) R_M' (Linear regression Plot): Consider $R_M = R_M' \times C_Z/C_Y$, the conversion of equation $R_M = mR_B + b$ leads to:

$R_B = (C_Z \times R_M') / (C_Y \times m) - b/m$

Let $m' = C_Z / (C_Y \times m)$ and $b' = - b/m$, we have:

$R_B = m'R_M' + b'$

The standard uncertainty of R_M' was calculated using the following equation:

$$u_{R_M} = \frac{1}{m'} \times s_{y/x} \times \sqrt{\frac{1}{N} + \frac{1}{n} + \frac{\left(R_B - \overline{R_{Bc}}\right)^2}{m'^2 \sum_{i=1}^{n} (R_{Mci} - \overline{R_{Mc}})^2}} \qquad (4)$$

Where,

$s_{y/x}$ = standard deviation of the regression

R_B = peak area ratio of sample blend

$\overline{R_{Bc}}$ = average peak area ratio of calibration blends

n = number of calibration blends used for the linear regression plot

N = injection time for each sample

R_{Mci} = isotope mass ratio in calibration blends

$\overline{R_{Mc}}$ = average isotope mass ratio in calibration blends

(4) F_{SV}: The standard deviation of the mean of results from different subsamples.

(5) F_{SDS}: Factor representing any bias in protein separation using SDS-PAGE.

(6) F_{PV}: The standard deviation of the mean of results from different signature peptides.

The combined standard uncertainty was calculated using the equation below:

$$u = \sqrt{\sum_i c_i^2 {u_{xi}}^2} \qquad (5)$$

Where,

u = combined standard uncertainty

c_i = sensitivity coefficient of each component

u_{xi} = standard uncertainty of each component

The full uncertainty budget is given in the Table below for total-GH:

	x	u_{xi}	u_{xi}/x	c_i	$c_i^2 . u_{xi}^2$	Contribution
M_X (g)	0.24688	0.000085	0.034%	57.1	2.348×10^{-5}	0.0035%
M_Y (g)	0.030056	0.000022	0.073%	469.1	0.000106	0.016%
C_Z (ng/g)	27.33	0.31	1.13%	0.52	0.02538	3.78%
R_M'	3.510	0.086	2.44%	4.02	0.11850	17.64%
F_{SV} (ng/g)	14.10	0.60	4.24%	1.00	0.35721	53.17%
F_{SDS} (ng/g)	14.10	0.28	2.00%	1.00	0.07950	11.83%
F_{PV} (ng/g)	14.10	0.30	2.14%	1.00	0.09106	13.56%

The full uncertainty budget is given in the Table below for 22kDa-GH:

	x	u_{xi}	u_{xi}/x	c_i	$c_i^2 . u_{xi}^2$	Contribution
M_X (g)	0.24688	0.000085	0.034%	52.4	1.979E-05	0.0033%
M_Y (g)	0.030056	0.000022	0.073%	430.6	8.910E-05	0.0149%
C_Z (ng/g)	27.33	0.31	1.13%	0.47	0.02139	3.59%
R_M'	3.222	0.084	2.61%	4.02	0.11398	19.11%
F_{SV} (ng/g)	12.94	0.58	4.49%	1.00	0.33835	56.74%
F_{SDS} (ng/g)	12.94	0.26	2.00%	1.00	0.06700	11.24%
F_{PV} (ng/g)	12.94	0.24	1.82%	1.00	0.05547	9.30%

Report of results

CCQM-K177
“Human Growth Hormone in serum: The determination of the mass fraction of total human growth hormone in serum”

Participating Laboratory

Institute: National Metrology Institute of Japan, NMIJ/AIST

Reporting date: Sep 22. 2022

Contact person: Tomoya Kinumi

E-mail: t.kinumi@aist.go.jp

Overall Results

Mass fraction of total-GH[1] in serum (mean of single results from replicate determinations in ng/g)	Combined Standard Uncertainty (ng/g)	Coverage Factor (*k*)	Expanded Uncertainty (ng/g)
12.2	**1.1**	**2**	**2.1**

optional:

Mass fraction of 22kDa-GH[1] in serum (mean of single results from replicate determinations in ng/g)	Combined Standard Uncertainty (ng/g)	Coverage Factor (*k*)	Expanded Uncertainty (ng/g)

[1] The molar mass of 22kDa-GH is 22124.8 g/mol, also to be used with total-GH measurements.

Single results

Signature peptide (e.g. T12…)	Mass fraction of total-GH in aliquot 1 of sample (ng/g)	Mass fraction of total-GH in aliquot 2 of sample (ng/g)	Mass fraction of total-GH in aliquot 3 of sample (ng/g)
T12	**11.7**	**11.8**	**13.0**
Signature peptide (e.g. T4, T6)	Mass fraction of 22kDa-GH in aliquot 1 of sample (ng/g)	Mass fraction of 22kDa-GH in aliquot 2 of sample (ng/g)	Mass fraction of 22kDa-GH in aliquot 3 of sample (ng/g)

Analytical conditions

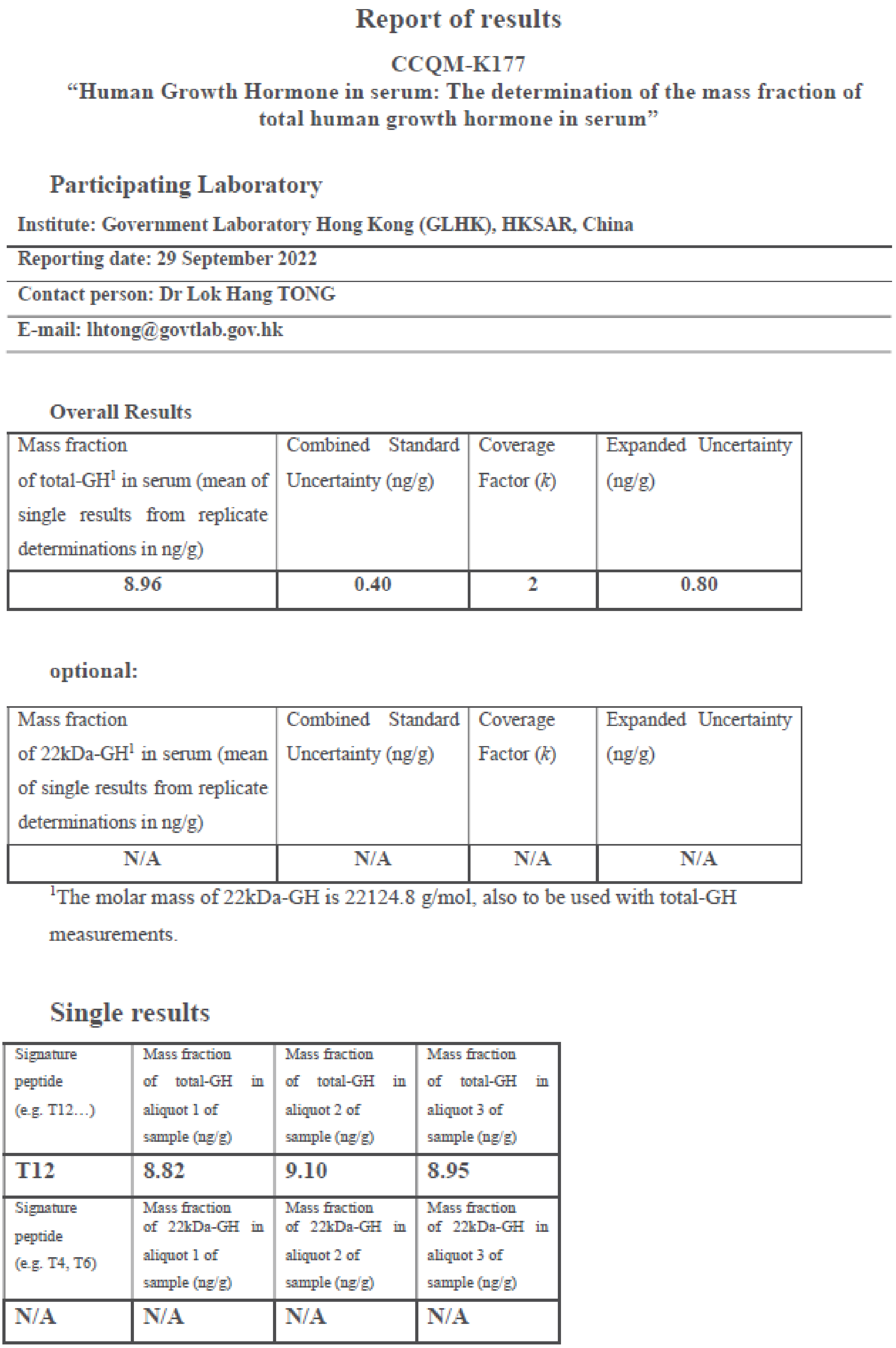
Sample treatment

Sample amount used for analysis (mg)	~0.7 g for each sub-sample
Brief description of the measurement (e.g. preparation of sample and calibrator, digestion, method and instrumentation used for purification of the analyte)	The sample preparation was performed according to the PTB' protocol. 1st chromatography: Column: Jupiter Proteo 90 A C12, (150 x 4.6 mm id, 4 μm, Phenomenex) Mobile phase (acidic) A: 0.1 %TFA/water, B 0.1 % TFA/acetonitrile Gradient program: 1 – 80 %B in 65 min, Flow: 1 mL/min 2nd chromatography: Column: Jupiter 300 A, C4 (250 x 4.6 mm id, 5 μm, Phenomenex) Mobile phase (basic) A: 0.05 % ammonium formate/water (pH 8), B 0.05 % ammonium formate/ 80 % acetonitrile (pH 8), Gradient program: 2 – 50 %B in 60 min, Flow: 1 mL/min Injection: 50 μL per injection, fractionation was done 6 times for each sub-sample.

Instrumentation and MS-acquisition

Instrumentation used (e.g., type of liquid-chromatograph and mass spectrometer, manufacturer, important instrument parameters, analytical technique used: LC-MS or LC-MS/MS etc.	LC-MS (QTOF) system was used. LC: Nexera high-pressure gradient system (Shimadzu) MS: maXis II (Bruker) The data was acquired by three repeated measurements.
Indicate signature peptides of total-GH /22kDa-GH and MS-acquisition parameters (ion/MRM, collision energy)	Signature peptide measured by extracted ion chromatography: T12, *m/z* 387.1936 (doubly charged ion) for natural, *m/z* 392.1787 (doubly charged ion) for isotopically labeled peptide.

Calibration method/design used	T12 peptide: one-point double ID calibration
Chromatographic Column (i.e., type and manufacturer)	SunShell C18 (150 x 2.1 mm id, 2.6 µm, ChromaNik Technologies)
Chromatographic Conditions (eluents, gradient and flow rate)	A: 0.1 % Formic acid/water, B: 0.1 % Formic acid/acetonitrile Gradient program: 1 – 8 %B in 30 min, Flow 0.25 mL/min
Measurement equation used to determine the mass fraction of total-GH/22kDa-GH in serum	According to one-point calibration
Uncertainty budget (please list all components and their contribution to the combined standard uncertainty)	Following four uncertainty components were considered. Uncertainty component (relative, %) Between digestion 5.31 Repeat measurement 4.90 Calibration curve 4.38 hGH reference material 1.10 Combined uncertainty (%) 8.52 Concentration (ng/g) 12.2 Standard uncertainty (ng/g) 1.1 Expanded uncertainty (ng/g, *k* = 2) 2.1
Additional Comments or Observations	



Report of results

CCQM-K177
"Human Growth Hormone in serum: The determination of the mass fraction of total human growth hormone in serum"

Participating Laboratory

Institute: Government Laboratory Hong Kong (GLHK), HKSAR, China

Reporting date: 29 September 2022

Contact person: Dr Lok Hang TONG

E-mail: lhtong@govtlab.gov.hk

Overall Results

Mass fraction of total-GH[1] in serum (mean of single results from replicate determinations in ng/g)	Combined Standard Uncertainty (ng/g)	Coverage Factor (*k*)	Expanded Uncertainty (ng/g)
8.96	**0.40**	**2**	**0.80**

optional:

Mass fraction of 22kDa-GH[1] in serum (mean of single results from replicate determinations in ng/g)	Combined Standard Uncertainty (ng/g)	Coverage Factor (*k*)	Expanded Uncertainty (ng/g)
N/A	**N/A**	**N/A**	**N/A**

[1] The molar mass of 22kDa-GH is 22124.8 g/mol, also to be used with total-GH measurements.

Single results

Signature peptide (e.g. T12…)	Mass fraction of total-GH in aliquot 1 of sample (ng/g)	Mass fraction of total-GH in aliquot 2 of sample (ng/g)	Mass fraction of total-GH in aliquot 3 of sample (ng/g)
T12	**8.82**	**9.10**	**8.95**
Signature peptide (e.g. T4, T6)	Mass fraction of 22kDa-GH in aliquot 1 of sample (ng/g)	Mass fraction of 22kDa-GH in aliquot 2 of sample (ng/g)	Mass fraction of 22kDa-GH in aliquot 3 of sample (ng/g)
N/A	**N/A**	**N/A**	**N/A**

Analytical conditions

Sample treatment

Sample amount used for analysis (mg)	
Brief description of the measurement (e.g. preparation of sample and calibrator, digestion, method and instrumentation used for purification of the analyte)	Sample (250 mg) was spiked with U-^{15}N-GH, which was used as internal standard. Calibration mixtures containing GH (reference material) and U-^{15}N-GH in blank serum were prepared. Whole serum tryptic digestion of samples and calibration mixtures was followed by two-step clean-up of T12 using semi-preparative HPLC with eluents at low pH (step 1) and high pH (step 2). T12-extracts were analysed using LC-MS/MS.

Instrumentation and MS-acquisition

Instrumentation used (e.g., type of liquid-chromatograph and mass spectrometer, manufacturer, important instrument parameters, analytical technique used: LC-MS or LC-MS/MS etc.	**Analytical technique used:** LC-MS/MS System (Agilent 1290 Infinity II Series UHPLC coupled with SCIEX QTRAP 6500+)
Indicate signature peptides of total-GH /22kDa-GH and MS-acquisition parameters (ion/MRM, collision energy)	**Signature peptide:** T12 **4 MS/MS transitions monitored:** Quantifying (*m/z* 387.3→531.2); Qualifying (*m/z* 387.3→243.1); Qualifying (*m/z* 387.3→215.1); Qualifying (*m/z* 387.3→660.0) **3 pairs of MRM:** 243.1/531.2; 215.1/531.1; 660.0/531.2 **Collision energy:** 15 eV
Calibration method/design used	Calibration curve
Chromatographic Column (i.e., type and manufacturer)	Aeris™ PEPTIDE XB-C18, 3.6 µm 250 × 2.1 mm

<table>
<tr>
<td>Chromatographic Conditions
(eluents, gradient and flow rate)</td>
<td>
Eluents:
Mobile phase A: 0.1 % HCOOH in water (v/v)
Mobile phase B: 0.1 % HCOOH in acetonitrile (v/v)
Gradient:
<table>
<tr><th>Time (min)</th><th>Mobile phase A</th><th>Mobile phase B</th></tr>
<tr><td>0</td><td>100</td><td>0</td></tr>
<tr><td>3</td><td>100</td><td>0</td></tr>
<tr><td>40</td><td>96.5</td><td>3.5</td></tr>
<tr><td>45</td><td>96.5</td><td>3.5</td></tr>
<tr><td>47.4</td><td>0</td><td>100</td></tr>
<tr><td>47.5</td><td>99</td><td>1</td></tr>
<tr><td>49.9</td><td>0</td><td>100</td></tr>
<tr><td>50</td><td>99</td><td>1</td></tr>
<tr><td>52.4</td><td>0</td><td>100</td></tr>
<tr><td>52.5</td><td>100</td><td>0</td></tr>
<tr><td>70</td><td>100</td><td>0</td></tr>
</table>
Flow rate: 0.2 mL/min
</td>
</tr>
<tr>
<td>Measurement equation used to determine the mass fraction of total-GH/22kDa-GH in serum</td>
<td>
The amount of growth hormone in sample (<i>Spl_Amt</i>) in ng is calculated using the following equation:
$$Spl\_Amt = Amt_{IS} \times \left\{ \frac{\frac{A_{spl}}{A_{IS}} - b}{m} \right\}$$
where
Spl_Amt = amount of T12 found in sample in ng
Amt_{IS} = amount of labelled standard T12* in sample in ng
A_{spl} = peak area of T12 in sample
A_{IS} = peak area of the corresponding labelled standard T12* in sample
b = <i>y</i>-intercept
m = slope

The mass fraction of growth hormone in sample (C_{Sample}) in ng/g is calculated as follows:
$$C_{sample} = \frac{Spl\_Amt}{W_{sample}}$$
where
Spl_Amt = amount of T12 found in sample in ng
W_{sample} = sample used in g
</td>
</tr>
</table>

<table>
<tr>
<td>Uncertainty budget (please list all components and their contribution to the combined standard uncertainty)</td>
<td>The uncertainty budget consists of three major contributions: i) purity of the given aliquot of serum containing 22kDa-GH (<i>i.e.</i> [u(std)]), ii) method precision (<i>i.e.</i> [u(pres)]) and iii) method bias (<i>i.e.</i> [u(bias)]).
<table>
<tr><th><i>Description</i></th><th><i>Value x</i></th><th><i>Std. Unc.</i></th><th><i>Rel. Std. Unc. u(x)</i></th></tr>
<tr><td>Reference Material [u(std)]</td><td>1</td><td>0.011000</td><td>0.011000</td></tr>
<tr><td>Precision [u(pres)]</td><td>1</td><td>0.024821</td><td>0.024821</td></tr>
<tr><td>Method Bias [u(bias)]</td><td>1</td><td>0.035544</td><td>0.035544</td></tr>
<tr><td>Combined Rel. Std. Unc.</td><td></td><td>0.044726</td><td></td></tr>
<tr><td>Rel. Expanded Unc. (U)</td><td></td><td>0.089452</td><td></td></tr>
</table>
</td>
</tr>
<tr>
<td>Additional Comments or Observations</td>
<td>Nil</td>
</tr>
</table>

Report of results

CCQM-K177

"Human Growth Hormone in serum: The determination of the mass fraction of total human growth hormone in serum"

Participating Laboratory

Institute:	
Reporting date: 20.09.2022	
Contact person: Cristian Arsene	
E-mail: christian.arsene@ptb.de	

Overall Results

Mass fraction of total-GH[1] in serum (mean of single results from replicate determinations in ng/g)	Combined Standard Uncertainty (ng/g)	Coverage Factor (*k*)	Expanded Uncertainty (ng/g)
9.51	**0.24**	**2.0**	**0.48**

Mass fraction of 22kDa-GH[1] in serum (mean of single results from replicate determinations in ng/g)	Combined Standard Uncertainty (ng/g)	Coverage Factor (*k*)	Expanded Uncertainty (ng/g)
8.13	**0.21**	**2.0**	**0.43**

[1] The molar mass of 22kDa-GH is 22124.8 g/mol, also to be used with total-GH measurements.

Single results

Signature peptide (e.g. T12…)	Mass fraction of total-GH in aliquot 1 of sample (ng/g)	Mass fraction of total-GH in aliquot 2 of sample (ng/g)	Mass fraction of total-GH in aliquot 3 of sample (ng/g)	Mass fraction of total-GH in aliquot 4 of sample (ng/g)	Mass fraction of total-GH in aliquot 5 of sample (ng/g)	Mass fraction of total-GH in aliquot 6 of sample (ng/g)
T12	**9.31**	**9.86**	**9.57**	**9.24**	**9.66**	**9.41**
Signature peptide (e.g. T4, T6)	Mass fraction of 22kDa-GH in aliquot 1 of sample (ng/g)	Mass fraction of 22kDa-GH in aliquot 2 of sample (ng/g)	Mass fraction of 22kDa-GH in aliquot 3 of sample (ng/g)	Mass fraction of 22kDa-GH in aliquot 4 of sample (ng/g)	Mass fraction of 22kDa-GH in aliquot 5 of sample (ng/g)	Mass fraction of 22kDa-GH in aliquot 6 of sample (ng/g)
T6	**7.91**	**8.18**	**7.92**	**7.93**	**8.41**	**8.45**

Analytical conditions

Sample treatment

Sample amount used for analysis (mg)	300 mg

Brief description of the measurement (e.g. preparation of sample and calibrator, digestion, method and instrumentation used for purification of the analyte)	Working solutions of 22 kDa-GH and U-^{15}N-22 kDa-GH in serum were prepared from stock solutions. For preparation, blank serum (4692.58 mg) was added to 30.824 mg of GH-stock solution. Aliquots of this solution were sent to participants. This solution was further diluted for GH-analysis in PTB`s laboratory: to an aliquot (e.g. 33.865 mg) blank serum (e.g. 2025.853 mg) was added. A working solution of U-^{15}N-22 kDa-GH in serum was prepared as follows: blank serum (1739.753 mg) was added to 18.675 mg of U-^{15}N-22 kDa-GH - stock solution. Aliquots of this solution were sent to participants. This solution was further diluted for GH-analysis in PTB`s laboratory: to an aliquot (35.019 mg) blank serum (2040,246 mg) was added. **Sample preparation:** 300 mg of sample were spiked with 26.1 mg of the working solution of U-^{15}N-22 kDa-GH. **Calibrator for sample:** Mixture of 26.4 mg of 22 kDa-GH-working solution, 26.1 mg of U-^{15}N-22 kDa-GH-working solution and 270 mg blank serum. Digestion and purification of signature peptides (T6 and T12) using two-step liquid chromatography as described by Arsene et al. in Drug Testing and Analysis (2018), DOI: 10.1002/dta.2350 The purified T6 was alkylated with IPDOA-iodide before submission to analysis by LC-MS/MS as described in Drug Testing and Analysis (2018), (DOI: 10.1002/dta.2350)

Instrumentation and MS-acquisition

Instrumentation used (e.g., type of liquid-chromatograph and mass spectrometer, manufacturer, important instrument parameters, analytical technique used: LC-MS or LC-MS/MS etc.	Instrumentation and MS-acquisition as described in Drug Testing and Analysis (2018), DOI: 10.1002/dta.2350
Indicate signature peptides of total-GH /22kDa-GH and MS-acquisition parameters (ion/MRM, collision energy)	Signature peptides: T6 (YSFLQNPQTSLCFSESIPTPSNR) and T12 (LEDGSPR) MS-acquisition parameters were as described in Drug Testing and Analysis (2018), DOI: 10.1002/dta.2350
Calibration method/design used	One-point calibration using a calibrator as described above.
Chromatographic Column (i.e., type and manufacturer)	As described in Drug Testing and Analysis (2018), DOI: 10.1002/dta.2350
Chromatographic Conditions (eluents, gradient and flow rate)	As described in Drug Testing and Analysis (2018), DOI: 10.1002/dta.2350

Measurement equation used to determine the mass fraction of total-GH/22kDa-GH in serum	$C_{GH} = \frac{\text{CM ref}}{\text{M sample}} \times \frac{\text{M spike(sample)}}{\text{M spike(ref)}} \times \frac{\text{R ref - R M(ref)}}{\text{R M(sef) - R spike}} \times \frac{\text{R spike - R M(sample)}}{\text{R M(sample) - R sample}}$ **C_{GH}** : concentration of GH in sample (mol/g) **C** ref : concentration of GH-working solution (mol/g) **M** ref : weight of GH-working solution (g) **CM r**ef : amount of substance of GH (mol), (**C** ref x **M** ref) **M** spike(sample): weight of working solution of U-^{15}N-GH, added to sample (g) **M** spike(ref): weight of working solution of U-^{15}N-GH, added to calibrator (g) **M** sample: weight of sample (g) **R** M(sample): isotope ratio of mixture "sample + U-^{15}N-GH", with **R** M(sample)= $FL_{\text{labeled T6}}/FL_{T6}$ ($FL_{\text{labeled T12}}/FL_{T12}$, FL= peak area in XIC **R** M(ref): isotope ratio of mixture "calibrator + U-^{15}N-GH", with **R** M(ref)= $FL_{\text{labeled T6}}/FL_{T6}$ ($FL_{\text{labeled T12}}/FL_{T12}$) **R** sample : isotope ratio(natural) of GH in sample (R sample = 0) **R** ref : isotope ratio (natural) of reference material (GH), R ref = 0 **R** spike : isotope ratio of U-^{15}N-GH **C** spike : concentration of U-^{15}N-GH-working solution (mol/g) Mass fraction of 22 kDa GH in serum: $w_{GH} = C_{GH}$*22124.8 g/mol

Uncertainty budget (please list all components and their contribution to the combined standard uncertainty)	- standard uncertainty of determination of the mass fraction (w_{GH}) : 0.095 ng/g (total-GH determination); 0.100 ng/g (22kDa-GH determination) - standard uncertainty contribution of value-assignment of 22kDa-GH reference solution used for preparation of GH-stock solution in serum: 0.100 ng/g (total-GH determination); 0.089 ng/g (22kDa-GH determination) - standard uncertainty of weighing: 0.007 ng/g (total-GH determination); 0.006 ng/g (22kDa-GH determination) - unknown factor for systematic unidentified discrepancies including sample preparation and LC-MS/MS interferences, estimated value for sample: 0.190 ng/g (total-GH determination); 0.160 ng/g (22kDa-GH determination) - standard uncertainty of molecular mass of 22kDa-GH:0.000016 combined standard uncertainty: $\mathrm{u_c} = \sqrt{0.095^2 + 0.100^2 + 0{,}190^2 + +0{,}007^2 + 0.000016^2}$ (total-GH determination) $\mathrm{u_c} = \sqrt{0.100^2 + 0.089^2 + 0{,}160^2 + 0.006^2 + 0{,}000016^2}$ (22kDa-GH determination)
Additional Comments or Observations	

Report of results

CCQMK177

“Human Growth Hormone in serum: The determination of the mass fraction of total human growth hormone in serum”

Participating Laboratory

Institute: UMCG

Reporting date: 17.03.2022

Contact person: Bas Sleumer and MJ Vos

E-mail: SleumerBas@prahs.com

Overall Results SNLELLR

Sample No.	Mass fraction of total-GH[1] in serum (mean of single results from replicate determinations in ng/g)	Combined Standard Uncertainty (ng/g) STDEV	Coverage Factor (*k*)	Expanded Uncertainty (ng/g)
CCQM-K177	**10.6**	**0.242**		
rhGH serum sample 1	**10.3**	**0.279**		
rhGH serum sample 2	**15.3**	**0.258**		

Overall Results LHQLAFDTYQEFEEAYIPK

Sample No.	Mass fraction of 22kDa-GH[1] in serum (mean of single results from replicate determinations in ng/g)	Combined Standard Uncertainty (ng/g)	Coverage Factor (*k*)	Expanded Uncertainty (ng/g)
CCQM-K177	**9.66**	**0.232**		
rhGH serum sample 1 (CCQM-P164)	**10.5**	**0.146**		
rhGH serum sample 2 (CCQM-P164)	**16.0**	**0.486**		

[1]The molar mass of 22kDa-GH is 22124.8 g/mol, also to be used with total-GH measurements.

Single results

Signature peptide (e.g. T12) SNLELLR	Mass fraction of total-GH in aliquot 1 of sample 1 (ng/g)	Mass fraction of total-GH in aliquot 2 of sample 1 (ng/g)	Mass fraction of total-GH in aliquot 3 of sample 1 (ng/g)
CCQM-K177	**10.5**	**10.4**	**10.9**
rhGH serum sample 1	**10.2**	**10.6**	**10.0**
rhGH serum sample 2	**15.4**	**15.0**	**15.5**
Signature peptide (e.g. T4, T6) LHQLAFDTYQEFEEAYIPK	Mass fraction of 22kDa-GH in aliquot 1 of sample 2 (ng/g)	Mass fraction of 22kDa-GH in aliquot 2 of sample 2 (ng/g)	Mass fraction of 22kDa-GH in aliquot 3 of sample 2 (ng/g)
CCQM-K177	**9.44**	**9.63**	**9.90**
rhGH serum sample 1	**10.4**	**10.6**	**10.4**
rhGH serum sample 2	**16.4**	**15.5**	**16.0**

Single results

GH STD (297.17 fmol/mg) diluted and converted to 41.0 ng/g that it will fit within the calibration curve

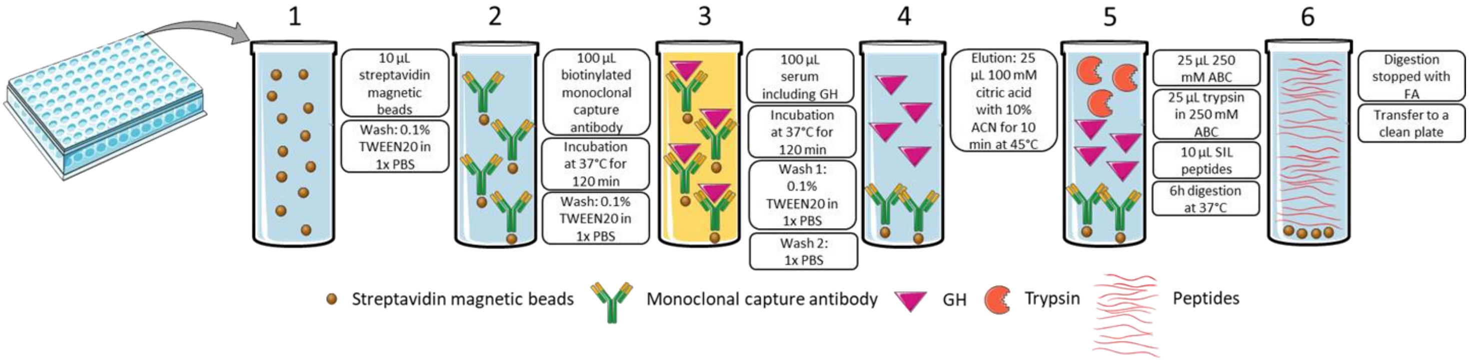
Signature peptide (e.g. T12) SNLELLR	Mass fraction of total-GH in aliquot 1 of sample 1 (ng/g)	Mass fraction of total-GH in aliquot 2 of sample 1 (ng/g)	Mass fraction of total-GH in aliquot 3 of sample 1 (ng/g)	Mass fraction of total-GH **mean** sample 1 (ng/g)	Factor
GH STD	**37.5**	**37.3**	**36.1**	**37.0**	**1.11**
Signature peptide (e.g. T4, T6) LHQLAFDTYQEFEEAYIPK	Mass fraction of 22kDa-GH in aliquot 1 of sample 2 (ng/g)	Mass fraction of 22kDa-GH in aliquot 2 of sample 2 (ng/g)	Mass fraction of 22kDa-GH in aliquot 3 of sample 2 (ng/g)	Mass fraction of total-GH **mean** sample 1 (ng/g)	
GH STD	**37.5**	**36.1**	**35.3**	**36.3**	**1.13**

Analytical conditions

Sample treatment

Sample amount used for analysis (mg)	100 µL (Density serum 1.0269)
Brief description of the measurement (e.g. preparation of sample and calibrator, digestion, method and instrumentation used for purification of the analyte)	calibration samples in rat plasma at 0.500, 1.00, 2.50, 5.00, 10.0, 25.0, 40.0 and 50.0 ng/mL prepared with a stock solution Growth Hormone Human Recombinant (Cat. No. CYT-202) from Prospec Protein Specialists (Ness-Ziona, Israel). SIL peptides used as internal standard

Workflow of immunoprecipitation of GH



Instrumentation and MS-acquisition

<table>
<tr><td>Instrumentation used (e.g., type of liquid-chromatograph and mass spectrometer, manufacturer, important instrument parameters, analytical technique used: LC-MS or LC-MS/MS etc.</td><td>M-class UPLC system coupled to a Xevo TQ-S triple-quadrupole mass spectrometer (Waters, Milford, MA, USA).</td></tr>
<tr><td>Indicate signature peptides of 22kDa-GH and MS-acquisition parameters (ion/MRM, collision energy)</td><td>
<table>
<tr><th>Peptide</th><th>Q1 m/z</th><th>Q3 m/z</th><th>Collision energy (V)</th></tr>
<tr><td>SNLELLR</td><td>422.7
[M+H]$^+$</td><td>643.4
(y5$^+$)</td><td>14</td></tr>
<tr><td>SNLELLR-SIL</td><td>427.8
[M+H]$^+$</td><td>653.4
(y5$^+$)</td><td>14</td></tr>
<tr><td>LHQLAFDTYQEFEEAYIPK</td><td>781.4
[M+2H]$^{2+}$</td><td>1050.0
(b17^{+2})</td><td>15</td></tr>
<tr><td>LHQLAFDTYQEFEEAYIPK-SIL</td><td>784.1
[M+2H]$^{2+}$</td><td>1050.0
(b17^{+2})</td><td>15</td></tr>
</table>
</td></tr>
<tr><td>Calibration method/design used</td><td>1/xx</td></tr>
<tr><td>Chromatographic Column
(i.e., type and manufacturer)</td><td>100 x 1.0 mm (particle size 1.6 µm, pore size 100 Å) Luna Omega C18 column (Phenomenex, CA, USA)</td></tr>
<tr><td>Chromatographic Conditions
(eluents, gradient and flow rate)</td><td>Mobile phase A consisted of 0.1% formic acid in water and mobile phase B was 0.1% formic acid in acetonitrile. Gradient elution was performed using the following profile at a flowrate of 80 µL/min: 0.0–6.0 min: 13–16% B; 6.1–11 min: 20–25% B; 11.1-14: 95% B; 14.1–16 min: 13% B. The injection volume was 8 µL.</td></tr>
<tr><td>Measurement equation used to determine the mass fraction of 22kDa-GH in serum</td><td></td></tr>
</table>

Uncertainty budget (please list all components and their contribution to the combined standard uncertainty)	
Additional Comments or Observations	