

This supporting information file shows the method validation results

1) Matrix effect

A total of 7 urine samples with creatinine levels ranging from 1.1 to 59.1 mmol/L were fortified with Gb₃ (2.5 µg/50 mL urine).

Each urine matrix was prepared in triplicate and analyzed in duplicate.

The difference between the highest and the lowest Gb₃ signal detected was measured to determine the matrix effect.

Matrix	Creatinine mmol/L	Gb ₃ area	Gb ₃ (C17:0) area	Ratio Gb ₃ /Gb ₃ (C17:0) area/area
1	1.1	197871	75857	2.60
2	2.9	201249	75253	2.66
3	4.6	204478	75364	2.70
4	9.7	209089	77511	2.65
5	16.7	217502	77542	2.76
6	21.0	217450	75865	2.59
7	59.1	199363	73271	2.61
Mean	na	206715	75809	2.65
(max-min)%	na	9.5	5.6	6.5

2) Limit of detection (LOD) and limit of quantification (LOQ)

The first point of the Gb₃ calibration curve (P1) was analyzed five times .

The standard deviation of the Gb₃ concentration measured was evaluated.

LOD and LOQ were respectively defined as 3 and 10 times this standard deviation.

	Gb ₃ µg/mL urine
LOD	0.007
LOQ	0.023

3) Intraday precision

The 3 quality controls (healthy control (Ctrl-), positive control heterozygous (Ctrl+ (Hetero)), and positive control hemizygous (Ctrl+ (Hemi)) were prepared in quintuplicate and analyzed the same day.

The coefficient of variation (CV%) was measured.

	Gb ₃	
	mg/mol Creat.	CV%
Ctrl-	4.5	6.6
Ctrl+ (Hetero)	25.3	4.5
Ctrl+ (Hemi)	387.5	6.3

4) Interday precision

The 3 quality controls (healthy control (Ctrl-), positive control heterozygous (Ctrl+ (Hetero)), and positive control hemizygous (Ctrl+ (Hemi)) were prepared in triplicate on five different days. The coefficient of variation (CV%) was measured. sd = standard deviation.

Day	Gb ₃			Creatinine			Gb ₃ /Creatinine		
	Ctrl- µg/mL	Ctrl+ (Hetero) µg/mL	Ctrl+ (Hemi) µg/mL	Ctrl- mmol/L	Ctrl+ (Hetero) mmol/L	Ctrl+ (Hemi) mmol/L	Ctrl- mg/mol Creat.	Ctrl+ (Hetero) mg/mol Creat.	Ctrl+ (Hemi) mg/mol Creat.
1	0.052	0.229	3.528	11.41	9.05	9.10	4.53	25.25	387.45
2	0.067	0.273	3.980	10.24	8.40	9.10	6.57	32.47	437.31
3	0.059	0.260	4.452	9.93	8.21	9.02	5.89	31.62	493.71
4	0.057	0.252	3.938	10.69	8.61	9.67	5.33	29.21	407.08
5	0.0441	0.2208	3.5680	10.46	8.54	9.14	4.22	25.85	390.20
mean	0.056	0.247	3.893	10.54	8.56	9.21	5.31	28.88	423.15
sd	0.0	0.0	0.4	0.6	0.3	0.3	0.97	3.27	44.15
CV%	15.4	8.8	9.6	5.3	3.7	2.9	18.2	11.3	10.4

5) Interday accuracy

The control urine used as matrix for the calibration curve was fortified with Gb₃ at 3 different levels and analyzed in triplicate on 5 different days.

Creatinine solutions in water were prepared at 3 different levels and analyzed in triplicate on 5 different days.

The standard stock solutions used to prepare the QCs were different from those used to prepare the calibration curves.

The bias% between the measured and the theoretical concentrations were calculated.

Day	Gb ₃			Creatinine		
	LQC (0.075 µg/mL)	MQC (1.5 µg/mL)	HQC (10 µg/mL)	LQC (0.2 mmol/L)	MQC (5.0 mmol/L)	HQC (20 mmol/L)
	Bias%	Bias%	Bias%	Bias%	Bias%	Bias%
1	-7.6	2.9	7.2	8.9	3.7	-0.2
2	8.0	17.3	13.1	8.6	2.5	-1.6
3	-30.0	2.1	6.9	7.3	2.4	-2.2
4	-15.6	6.2	-7.0	-13.3	2.6	-1.0
5	-37.7	1.4	-16.0	4.8	1.3	-3.4
Mean	-16.6	6.0	0.8	3.3	2.5	-1.7
Min	-37.7	1.4	-16.0	-13.3	1.3	-3.4
Max	8.0	17.3	13.1	8.9	3.7	-0.2

Note: The bias% measured for the Gb₃ LQC was relatively high on some occasions. We think that this could reflect an incompletely homogeneous distribution of Gb₃ when it was spiked at a low concentration in urine, even after a long vortexing time. This variability in the results was not observed for the analysis of Gb₃ in the negative control urine, which also has a low Gb₃ concentration (See section 4).

6) Linearity of the calibration curves

Both calibration curves (Gb₃ and creatinine) were prepared on 9 different days and the correlation factor (R²) was measured.

Day	Gb ₃ R ²	Creatinine R ²
1	0.9991	0.9995
2	0.9995	0.9997
3	0.9980	0.9997
4	0.998	0.9999
5	0.9973	0.9996
6	0.9977	0.9996
7	0.9965	0.9998
8	0.9985	0.9998
9	0.9982	0.9998
Mean	0.9977	0.9998
Min	0.9965	0.9996
Max	0.9985	0.9999

7) Analytical measurement range

The inferior limit of the calibration curve linearity range was set to the lowest (non zero) point of the calibration curve. The superior limit of the analytical measurement range was defined as the highest concentration above the calibration curve range where the bias between the measured and the calculated concentration was <20%.

	Calibration curve range		Analytical measurement range	
	Inferior limit	Superior limit	Inferior limit	Superior limit
Creatinine (mmol/L)	0.1	30	0.1	60
Gb ₃ (µg/mL)	0.025	12	0.025	36

8) Sample stability

The stability of prepared samples was evaluated for 3, 5 and 12 days at room temperature (RT, 21°C), and 14 days at 4°C.

The stability of urine was evaluated for 2 days at RT, 7 days at 4°C and after 4 freeze/thaw cycles.

Results are expressed as bias% for the Gb₃/creatinine ratio between the stability point and the reference value (n = 3).

	Prepared samples				Urine		
	RT 3 days Bias%	RT 5 days Bias%	RT 12 days Bias%	4°C 14 days Bias%	RT 2 days Bias%	4°C 7 days Bias%	4 freeze/thaw cycles) Bias%
Ctrl-	-14.3	2.1	-19.6	-7.7	4.1	-5.0	-9.9
Ctrl+ (Hetero)	1.8	4.2	-4.4	4.5	3.7	5.6	-1.2
Ctrl+ (Hemi)	-1.8	1.3	-3.7	13.4	-3.4	2.4	-1.9

9) Adsorption of sample to glassware

Prepared samples were transferred three times into different glass tubes with different glass pipettes (n = 3). After each transfer, the sample was vortexed 10 s in the glass tube.

	Reference			3 transfers			Adsorption Gb ₃ /Creat. Bias%
	Creat. nM	Gb ₃ µg/mL	Gb ₃ /Creat. mg/mol Creat.	Creat. nM	Gb ₃ µg/mL	Gb ₃ /Creat. mg/mol Creat.	
Ctrl-	10.020	0.050	4.957	10.031	0.048	4.792	-3.3
Ctrl+ (Hetero)	8.231	0.301	36.583	8.236	0.268	32.571	-11.0
Ctrl+ (Hemi)	8.986	4.092	455.371	8.954	3.901	435.672	-4.3

10) Adsorption of sample to plasticware

Prepared samples were transferred three times into different plastic tubes with different plastic tips (n = 3). After each transfer, the sample was vortexed 10 s in the plastic tube.

	Reference			3 transfers			Adsorption Gb ₃ /Creat. Bias%
	Creat. nM	Gb ₃ µg/mL	Gb ₃ /Creat. mg/mol Creat.	Creat. nM	Gb ₃ µg/mL	Gb ₃ /Creat. mg/mol Creat.	
Ctrl-	10.041	0.060	5.992	10.044	0.060	5.941	-0.86
Ctrl+ (Hetero)	8.085	0.294	36.380	8.075	0.254	31.509	-13.39
Ctrl+ (Hemi)	8.996	3.728	414.378	9.010	3.867	429.170	3.57

11) Carryover evaluation

Injection of a blank sample (5% H₂O in methanol) just after the highest point of the calibration curve on four different days.

Day	Carryover(%)	
	Creatinine	Gb ₃
1	0	0.29
2	0	0.20
3	0	0.17
4	0.011	0.16
Mean	0.0028	0.20