HBM4EU science and policy for a healthy future

HORIZON2020 Programme
Contract No. 733032 HBM4EU

ICI / EQUAS REPORT

DINCH/round_03 (2019)

DINCH biomarkers in urine

Version / date of issue	1.2 / 27-08-2019
Organiser Coordinator	Wageningen Food Safety Research*, part of Wageningen University & Research, Akkermaalsbos 2, 6708 WB, Wageningen, The Netherlands * WFSR is the new name for RIKILT since 01.06.2019 Ingrid Elbers, ingrid.elbers@wur.nl
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Approved by:	Hans Mol (WFSR)

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Appendices

1 Summary

Within the frame of the HBM4EU project, an EQUAS study was organised on the determination of two DINCH biomarkers in urine. This was the second ICI/EQUAS round for this substance group within the HBM4EU program.

In total 14 laboratories were invited for this second round, of which 12 laboratories registered. Results were received from all 12 laboratories, located in 9 EU countries and the USA (see Appendix 1).

In May 2019, each participant received two burdened control materials of human urine, A and B (single tube each), containing DINCH biomarkers in the range 1-15 ng/ml.

Homogeneity assessment showed that both materials were sufficiently homogeneous for ICI/EQUAS testing. Previously conducted stability tests had shown that the biomarkers are stable when stored in the freezer and no significant loss of the biomarkers occurred during the course of the EQUAS test.

The proficiency of the laboratories was assessed through Z-scores calculated using the mean concentration as established by expert laboratories as assigned value, and a fixed fit-for-purpose relative target standard deviation (FFP-RSD_R) of 25%. Assigned values and Z-scores could be determined for both biomarkers in both test materials.

Two laboratories reported only one of the two biomarkers, all others determined both. The percentage of satisfactory Z-scores was around 80-100% for both biomarkers when including the results from the expert laboratories.

The characteristics and outcome of this EQUAS are summarized in Table 1.

Table 1. Summary table EQUAS results.

		Assigned value	study RSD_R[1)]	Z-scores			
Biomarker	Sample	(ng/ml)	%	No	satisfactory	questionable	unsatisfactory
OH-MINCH	A	1.09	41%	12	83%	17%	0%
	B	13.0	19%		100%	0%	0%
cx-MINCH	A	1.09	53%	10	80%	10%	10%
	B	8.30	15%		100%	0%	0%

[1)] interlaboratory relative standard deviation (robust RSD based on participants' results, excluding expert labs)

Compared to previous rounds, an improvement in laboratory performance was observed, indicating that the ICI/EQUAS exercises result in an overall improvement in the quality of DINCH biomarker analysis.

2 Introduction

Interlaboratory Comparison Investigations (ICI) and External Quality Assurance Schemes (EQUAS) are tools to access the proficiency of laboratories, and the comparability and reliability of analytical methods. Participation in ICI / EQUAS forms an integral part of quality control, in addition to initial and on-going in-house method validation.

This EQUAS study has been organised within the frame of HBM4EU as part of the Quality Assurance program for biomonitoring analyses, following protocols HBM4EU-SOP-QA-001 to 004 which are available through the HBM4EU website. Within HBM4EU, participation in ICI/EQUAS exercises is mandatory for laboratories that will analyse HBM4EU samples.

This report describes the outcome of the 3rd ICI/EQUAS round for DINCH in urine and was organised by Wageningen Food Safety Research (WFSR), part of Wageningen University & Research in the Netherlands (WFSR is the new name for RIKILT since 01.06.2019). WFSR is ISO/IEC 17043 accredited for organisation of proficiency tests, but the specific substances in this EQUAS study were outside the specified scope of accreditation.

The selection of the most relevant/feasible biomarkers for DINCH was previously done in WP9, and has been described in Deliverable report 9.2 v1.1. Based on this, two target biomarkers were included in the EQUAS for DINCH biomarker analysis (see Table 2).

Table 2. Biomarkers for DINCH* included in the EQUAS.

Biomarker	
OH-MINCH	cyclohexane-1,2-dicarboxylate-mono-(7-hydroxy-4-methyl)octyl ester
cx-MINCH	cyclohexane-1,2-dicarboxylate-mono-(7-carboxylate-4-methyl)heptyl ester

* Di-isononyl cyclohexane-1,2-dicarboylate

For this third round, the concentrations aimed at were between the median and 95th percentile concentrations as reported by [Correia-Sá 2017], (approx. 1-15 µg/L). Based on the information on LOQs provided by the participants during registration of this EQUAS (0.1 to 1 µg/L), determination of these levels should be feasible.

2.1 Confidentiality

In this report the identity of the participants and the information provided by them are treated as confidential. However, lab codes of the participants will be disclosed to the HBM-QAU for performance assessments.

3 Control material

3.1 Preparation of control material

For this EQUAS two control materials, A and B, were prepared, one aiming at concentrations in the range of approximately 2-3 ng/ml and one at 10-15 ng/ml. The control materials were prepared by blending aliquots of different burdened human urine samples. The burdened human urines were kindly provided by the Institute for Prevention and Occupational Medicine of the German Social Accident Insurance (IPA), with concentration estimates.

For blending, the selected materials were thawed, the appropriate volumes taken and mixed. The blend (approx. 500 ml) was centrifuged to remove any precipitates. Then the urine was aliquoted (4 ml portions) into coded polypropylene tubes with screwcap. The tubes were stored in the freezer (8th April 2019, <-18°C). Part of the tubes were stored at -80°C as reference for stability testing.

3.2 Homogeneity of control material

Homogeneity testing was done as described in HBM4EU-SOP-QA-002. Ten tubes of control material A and ten tubes of control material B were randomly selected from the freezer and sent to IPA for analysis. Each sample was analysed in duplicate. In brief, after thawing/mixing, an aliquot of the urine was taken, isotope labels of the biomarkers were added as internal standard, and a deconjugation step using *E. coli* β-glucuronidase was performed. The deconjugated urine was analysed by on-line SPE coupled to LC-MS/MS. The analysis results were sent to the organiser and processed according to the SOP using an Excel macro ("HBM4EU macro homogeneity test v1.xlsm"). The mean concentrations and relative standard deviations as obtained during homogeneity testing are presented in Table 3. The statistical evaluation of materials A and B for each of the biomarkers are provided in Appendix 2. It was concluded that homogeneity was adequate for both biomarkers in both control materials.

Table 3. Concentration of DINCH biomarkers as obtained during homogeneity testing (details see Appendix 2).

	material A		material B	
Biomarker	µg/L	RSD_r	µg/L	RSD_r
OH-MINCH	1.11	2%	13.5	2%
cx-MINCH	1.17	2%	8.60	2%

3.3 Stability of control material

At the time of drafting this report, no stability testing for the materials from this round had yet been done. However, stability testing from the previous rounds showed that both biomarkers were stable in urine when stored at -18°C in the freezer for at least five months.

4 Organisational details

4.1 Participants

Participants for this EQUAS study were laboratories from the HBM4EU consortium (including linked-third parties) that had been included as candidate laboratories for analyses in the frame of the HBM4EU project through WP9 (Task 9.2, Deliverable 9.3). A list of 14 eligible candidate laboratories was provided to WFSR. Invitation letters were sent by e-mail on 19th of April 2019 (see Appendix 3). For registration, each participant was asked to provide whether or not both target biomarkers were included in their scope of analysis, and the LOQs in µg/L (=ng/ml).

In total 12 laboratories from 9 EU countries and the USA registered. This included three expert laboratories, two from the HBM4EU consortium and one from the USA. Results were received from all laboratories (see Appendix 1).

4.2 Dispatch and instructions

Test materials (one tube A and one tube B, with unique codes, containing approx. 4 ml urine each, frozen conditions) were dispatched to the participants on 13th May 2019. The samples were packed in an insulation box with dry ice and sent by courier. Instructions and an "acknowledgement of receipt form" were included in the box and also sent by e-mail at the day of shipment (see Appendix 4). Participants were asked to check the content of the box upon receipt, to store the samples in the freezer, and to analyse the samples according to their routine method. The deadline for submission of results was 14th June 2019.

As in the 2nd round, special instructions were given to all laboratories regarding the transition to be used for quantification in the LC-MS/MS analysis, and the use of sufficiently wide acquisition windows to ensure that all isomer peaks of the biomarkers were included in the measurement (see also Appendices 2 and 3). This was done because DINCH biomarkers in burdened urine are isomeric mixtures which may result in multiple and/or broad peaks, and because the transition used for quantification may affect the analysis result. The very high variability of the results observed in the 1st round was attributed to this, and it was decided to harmonise on the quantifier transitions in order to improve interlaboratory precision.

Together with the instructions sent by email, also a request to provide detailed method information in an Excel file was sent to the participants. In this sheet, the participants were asked to specify the limit of quantification (LOQ) for each of the biomarkers. In addition, details on enzymatic deconjugation, cleanup, analysis technique, internal standards used, and precision data was asked for.

4.3 Deviations from ICI/EQUAS SOPs

For this 3rd round, the HBM4EU-QA-SOPs (version 2) were followed. There were no deviations from these SOPs.

5 Data evaluation

5.1 False positives and <LOQ

Classification of false positives and biomarkers reported as "<LOQ-value" or "not detected" is described in HBM4EU-SOP-QA-003. In this EQUAS there were no false positives and no non-detects. Therefore no further description is given here.

5.2 Assigned value

For EQUAS studies, the concentration as established by expert laboratories was used as assigned value. Expert laboratories were selected by the HBM4EU quality assurance unit. In this EQUAS round, a list of three laboratories was provided to the organiser, two from the HBM4EU consortium and one from the USA. The expert laboratories all agreed to collaborate. The expert laboratories received the same control material and instructions as the participants. However, instead of one test sample and single analysis, expert laboratories received six test samples to be analysed in duplicate. Upon receipt of their results and method information, the acceptability of the results for establishment of the expert value was verified. The following aspects were taken into account:

- precision (RSD_r) of the results provided by each expert lab.

- use of the isotopically labelled analogue as internal standard for each of the biomarkers analysed. For determination of the expert value, not using such internal standard was an exclusion criterion.

- use of the prescribed quantifier transitions for quantification (m/z 313>153 for OH-MINCH, m/z 327>173 for cx-MINCH). Not using the prescribed transitions was an exclusion criterion.

Next, the expert value was determined as described in HBM4EU-QA-001. In brief, using the individual means of the expert laboratories, the mean of the means was calculated and its relative uncertainty. The mean of means was used as assigned value when the relative uncertainty was below $0.7*\sigma_T$. When this condition was not met, and no outliers could be identified, then the uncertainty of the expert-derived mean was considered too high to be used as assigned value. The other requirement to be met was that the number of (remaining) individual expert means had to be at least three.

In case no expert value could be obtained, the consensus value derived from the combined results from both participants and expert laboratories could be investigated as an alternative option.

5.3 Target standard deviation (σ_T)

For calculation of the Z-scores, a fit-for-purpose relative target standard deviation (FFP-RSD_R) of 25% of the assigned value was used as target standard deviation for proficiency. This was the default indicated in HBM4EU-SOP-QA-003 and considered appropriate based on the outcome of the first two rounds.

5.4 ICI/EQUAS standard deviation (RSD_R)

To gain insight in the actual interlaboratory variability of the biomarkers determined in this study, the robust relative standard deviation (RSD_R) was calculated based on the participants' results, as described in HBM4EU-SOP-QA-003. For this the results of the expert laboratories were not included.

5.5 Z-scores

Z-scores were calculated according to SOP HBM4EU-SOP-QA-003.

$$Z = \frac{x - C}{\sigma_T} \qquad (1)$$

with: Z = Z-score for the submitted analysis result;
x = result submitted by the laboratory;
C = expert-assigned value;
σ_T = target standard deviation, here 0.25*C

In accordance with ISO 13528 and ISO 17043 and the deliverable D 9.4 "*The Quality Assurance/Quality Control Scheme in the HBM4EU project*, Z-scores are classified as presented in Table 4.

Table 4: Classification of Z-scores

$\lvert Z \rvert \leq 2$	Satisfactory
$2 < \lvert Z \rvert < 3$	Questionable
$\lvert Z \rvert \geq 3$	Unsatisfactory

6 Results and discussion

6.1 Results submitted by participants

In total 12 laboratories from nine EU countries and the USA (see appendix 1) agreed to participate in this EQUAS. All submitted results.

The scope of the laboratories and the LOQs for both biomarker as submitted together with the analysis results through the Excel method information sheet are provided in Appendix 5. Ten laboratories measured both DINCH biomarkers, two laboratories measured only OH-MINCH.

The LOQs varied from 0.05-1 ng/ml, but was generally around 0.2 ng/ml (see Appendix 5).

The individual analysis results of the laboratories are included in appendix 6.

Laboratories were asked to provide details on the method used for analysis. In general, the laboratories did not do any filtration/centrifugation after thawing the urine sample, added isotope label(s) to an aliquot of 0.2-3 ml urine, and adjusted the pH to values ranging from 4.5 to 6.5. All labs did an enzymatic deconjugation step, mostly using *E. Coli* β-glucuronidase (one lab used *Helix Pomatia* β-glucuronidase/aryl-sulfatase), at 37°C for 1.5-2.5 hours (in some cases overnight). In most cases the deconjugated urine was acidified and then extracted/preconcentrated using on-line or off-line SPE. The biomarkers were then measured using liquid chromatography with mass spectrometric detection (LC-MS/MS, electrospray ionisation in negative mode). In most cases, the laboratories used the isotopically labelled analogue of the biomarker as internal standard. All laboratories used the prescribed quantifier transitions for quantification (two participants did not provide the details).

In the method information sheet, the laboratories were also asked to provide existing precision data from (on-going) validation, i.e. repeatability, intermediate precision and measurement uncertainty. Seven out of the twelve laboratories provided this information (in some cases only on repeatability).

6.2 Assigned values and (target) standard deviations

The assigned value was the expert-assigned value as derived from replicate analysis of the control materials by three expert laboratories as described in 5.2. The repeatability of the results for each biomarker by each expert lab was very good (typically RSD_r <10%, 1x 16%). In all cases, the isotopically labelled analogue of the biomarker was used as internal standard, and the prescribed transition for quantification. The individual means of the expert labs were in good agreement with each other.

Expert-assigned values could be established for both biomarkers in both control materials. The assigned values and their uncertainties are included Appendix 6.

The target standard deviation used for determination of the Z-scores was 25% (0.25*C) (see 5.3 and 5.5). To verify how this fixed target value compares to the actual interlaboratory variability of the results, the relative standard deviation (study RSD_R, robust statistics) derived from the participants' results (excluding the results from the expert labs) were calculated. The RSD_R's are included in Appendix 6. They were high (41% and 53%) for the low concentrations, and good (15-19%) for the higher concentrations. The overall interlaboratory comparability improved compared to the 2nd round.

From the data, it was also verified to what extent the robust means of the participants deviated from the expert-values. These deviations were <20% for OH-MINCH. For cx-MINCH the robust mean (sample B) was 39% lower than the expert value.

6.3 Assessment of laboratory performance

Z-scores were calculated for the two biomarkers in both control materials. For each of the laboratories, the individual Z-scores for the biomarkers in both samples are provided in Appendix 6. A graphical representation of the individual Z-scores is shown in appendix 7.

A summary of number of laboratories that reported results, and the percentage of satisfactory, questionable, and unsatisfactory Z-scores is included in Table 1. The percentage of satisfactory Z-scores obtained was 80%-100%.

6.4 Conclusions and recommendations

In this 3rd ICI/EQUAS round on two DINCH biomarkers in urine, 12 laboratories (including three expert labs) registered and submitted results.

The interlaboratory variability of results was high at ~1 ng/ml, but good at 9-14 ng/ml. Compared to the second round, a further improvement in interlaboratory comparability has been made, and most Z-scores were satisfactory. Point of attention is the 39% difference between expert value and robust mean for cx-MINCH. Furthermore, as observed from the high RSD_R, the determination of both biomarkers at low level (1 ng/ml) is still challenging.

7 References

Analytical Methods Committee, 1989a, Robust statistics - How not to reject outliers Part 1. Basic concepts, Analyst, 114, 1693-1697.

Analytical Methods Committee, 1989b, Robust statistics - How not to reject outliers Part 2. Interlaboratory trials, Analyst, 114, 1699-1702.

Correia-Sá L, Schutze A, Norberto S, Calhau C, Domingues VF, Koch HM, Environment Int. 102 (2017) 79-86.

HBM4EU-SOP-QA-001 (version 2) "Organisation of Interlaboratory Comparison Investigations (ICI) and External Quality Assurance Schemes (EQUAS) of interlaboratory studies"

HBM4EU-SOP-QA-002 (version 2) "Preparation of control materials for Interlaboratory Comparison Investigations (ICI) and External Quality Assurance Schemes (EQUAS)"

HBM4EU-SOP-QA-003 (version 2) "Evaluation of results from Interlaboratory Comparison Investigations (ICI) and External Quality Assurance Schemes (EQUAS)"

HBM4EU-SOP-QA-004 (version 2) "Reporting of results of Interlaboratory Comparison Investigations (ICI) and External Quality Assurance Schemes (EQUAS)"

Note: the above mentioned SOPs can be found on the HBM4EU website: https://www.hbm4eu.eu/online-library/?mdocs-cat=mdocs-cat-1&mdocs-att=null

HBM4EU Deliverable 9.2 Prioritised list of biomarkers, matrices and analytical methods for the 1st prioritisation round of substances. https://www.hbm4eu.eu/deliverables/

ISO/IEC 17043:2010, Conformity assessment – General requirements for proficiency testing

ISO 13528, 2015, Statistical methods for use in proficiency testing by interlaboratory comparison.

Official Methods of Analysis Program Manual, 2002, Appendix D: Guidelines for Collaborative Study Procedures To Validate Characteristics of a Method of Analysis. Association Of Analytical Communities International. http://www.aoac.org/vmeth/Manual_Part_6.pdf.

Appendix 1. List of countries participating

Country	Number of laboratories participating
Belgium	2
Czech Republic	2
Denmark	1
Germany	1
Greece	1
Hungary	1
Norway	1
Slovakia	1
Sweden	1
USA	1

Appendix 2. Homogeneity data

	Control material A			Control material A	
	OH-MINCH			cx-MINCH	
	replicate-1	replicate-2		replicate-1	replicate-2
1	1.14	1.08		1.15	1.14
2	1.09	1.15		1.17	1.16
3	1.11	1.12		1.17	1.16
4	1.09	1.13		1.19	1.16
5	1.11	1.06		1.15	1.16
6	1.1	1.08		1.16	1.17
7	1.10	1.09		1.18	1.2
8	1.10	1.12		1.15	1.2
9	1.09	1.11		1.14	1.16
10	1.13	1.11		1.14	1.2
grand mean	1.106			1.166	
Stdev	0.022			0.020	
VC%	2%			2%	
Cochran's test					
C	0.275			0.434	
Ccrit	0.602			0.602	
C < Ccrit →	No outliers detected			No outliers detected	
target σ_{FFP}	0.276			0.291	
s_x =	0.0123			0.0132	
s_w =	0.0256			0.0204	
s_s =	0.0000			0.0000	
critical=0.3σ_{FFP}	0.0829			0.0874	
s_s < critical?	Homogeneity adequate			Homogeneity adequate	
s_w< 0.5*σ_{FFP}?	Method suited			Method suited	

	Control material B			Control material B	
	OH-MINCH			cx-MINCH	
	replicate-1	replicate-2		replicate-1	replicate-2
1	13.55	13.21		8.34	8.68
2	13.47	13.12		8.56	8.19
3	13.59	13.01		8.62	8.68
4	14.31	13.59		8.63	8.86
5	13.68	13.92		8.75	8.83
6	13.63	13.42		8.52	8.4
7	13.75	13.63		8.64	8.65
8	13.61	13.33		8.74	8.6
9	13.46	13.59		8.81	8.63
10	13.09	13.23		8.29	8.55
grand mean	13.510			8.599	
Stdev	0.303			0.179	
VC%	2%			2%	
Cochran's test					
C	0.392			0.305	
Ccrit	0.602			0.602	
C < Ccrit →	No outliers detected			No outliers detected	
target σ_{FFP}	3.377			2.150	
s_x =	0.2454			0.1459	
s_w =	0.2573			0.1499	
s_s =	0.1647			0.1003	
critical=0.3σ_{FFP}	1.0132			0.6449	
s_s < critical?	Homogeneity adequate			Homogeneity adequate	
s_w< 0.5*σ_{FFP}?	Method suited			Method suited	

Appendix 3. Copy of letter of invitation

WAGENINGEN UNIVERSITY & RESEARCH

HBM4EU science and policy for a healthy future

Postbus 230 | 6700 AE WAGENINGEN The Netherlands

HBM4EU: Announcement / invitation to participate in ICI-EQUAS study DINCH/round_03

Title of ICI: DINCH biomarkers in urine

RIKILT

DATE
April 19, 2019

SUBJECT
Invitation for ICI-EQUAS study DINCH biomarkers in urine

POSTAL ADDRESS
Akkermaalsbos 2
6708 WB, Wageningen
The Netherlands

INTERNET
www.wur.nl/rikilt

CoC NUMBER
09095104

HANDLED BY
Ingrid Elbers

TELEPHONE
+31 317 481451

EMAIL
pt.rikilt@wur.nl

Dear colleague,

Within the frame of HBM4EU, RIKILT – Wageningen University & Research announces the second round of ICI/EQUAS for the determination of DINCH-biomarkers in urine. The aim of ICI/EQUAS exercises is to provide laboratories with an assessment of their analytical performance and reliability of their data in comparison with other laboratories and/or expert laboratories. This will aid in the quality improvement of analysis in human biomonitoring at each of the laboratories.

Participation is mandatory for laboratories (to be) involved in the analysis samples in the frame of HBM4EU.

1. Samples

- Two urine samples (approximately 2-4 ml) will be supplied for the analysis of DINCH biomarkers. See the registration form for potentially present biomarkers.
- The samples are expected to be sent by 13th May 2019. The distribution of the samples will be announced by e-mail.

2. Quantitative analysis

- The deadline for reporting the results is June 14th 2019.
- Results must be submitted via the electronic submission form (https://crlwebshop.wur.nl/apex/f?p=307:LOGIN) for which, if not already done so for the first round, each participant must register, as explained in the document "Instruction registration login account RIKILT Laboratory Quality Services".

3. Report

- A report of the proficiency test will be dispatched in July-August 2019.
- Results of the proficiency test will be presented anonymously.

4. Additional information

- The analysis results submitted by the laboratory and the performance derived from that will be treated confidentially, and will never be disclosed to third parties (government, accreditation bodies) except the HBM4EU QAU, without permission of the laboratory.

100 years 1918 — 2018

Wageningen Research Foundation/RIKILT is part of Wageningen University & Research. RIKILT carries out research into the safety and reliability of food and feed. RIKILT is ISO 17025 and ISO 17043 accredited (the accredited tests are described on www.rva.nl (no. L014 and R012).

Appendix 3. Copy of letter of invitation (continued)

DATE
April 19, 2019

PAGE
2 of 2

- Please take advantage of the feedback and suggestions for performance improvement provided to the participants during the Webex on 11th October 2018. Slides on this have been sent to most of you before, if you do not have them and would like to have it, please contact us.
- DINCH biomarkers are isomeric mixtures typically resulting in multiple and/or broad peaks in real samples, and the transition used for quantification affects the analysis result. For this reason the participants are asked to use the following transitions for quantification:
  OH-MiNP: m/z 313 > 153
  cx-MINCH: m/z 327 > 173
- Also, please ensure the acquisition windows for these compounds are sufficiently wide (at least from 2 min before until 2 min after the retention time of the analytical standard, depending on your specific LC method even longer) to ensure all peaks belonging to the biomarker are measured.

5. Costs

- For partners and linked-third parties of HBM4EU, participation is free of charge. Please note that the participant is responsible for custom clearance and associated costs if applicable.

6. Calender

- Deadline registration: 7th May 2019
- Distribution of samples: 13th May 2019
- Deadline submission of results: 14th June 2019

If you would like to participate, please fill out the registration form and send it to me before 7th May 2019 by e-mail (pt.rikilt@wur.nl). Please indicate on the registration form which biomarkers are within the scope of your method, and the estimated LOQ.

Hoping to welcome you for this ICI/EQUAS round,

Yours sincerely,

Ingrid Elbers (organiser proficiency test/ICI/EQUAS)
Hans Mol (scientific expert)

Appendix 3. Copy of letter of invitation (continued)

WAGENINGEN UNIVERSITY & RESEARCH

HBM4EU science and policy for a healthy future

Registration form EQUAS study DINCH/round_03 (RIKILT code 2019-06)

Contact person:	
E-mail:	
Username for RIKILT web application: (needed for reporting results)	
No username? Please register at:	Register (Ctrl + click)

Please indicate below the biomarkers that are within the scope of your method, and provide an estimate of your LOQ. We would appreciate your registration for both biomarkers.

Biomarkers included in the scope of your method (please tick)	Abbreviation	LOQ (ng/ml urine)
◻ cyclohexane-1,2-dicarboxylic mono hydroxyisononyl ester	OH-MINCH	
◻ cyclohexane-1,2-dicarboxylic mono carboxyisooctyl ester	cx-MINCH	

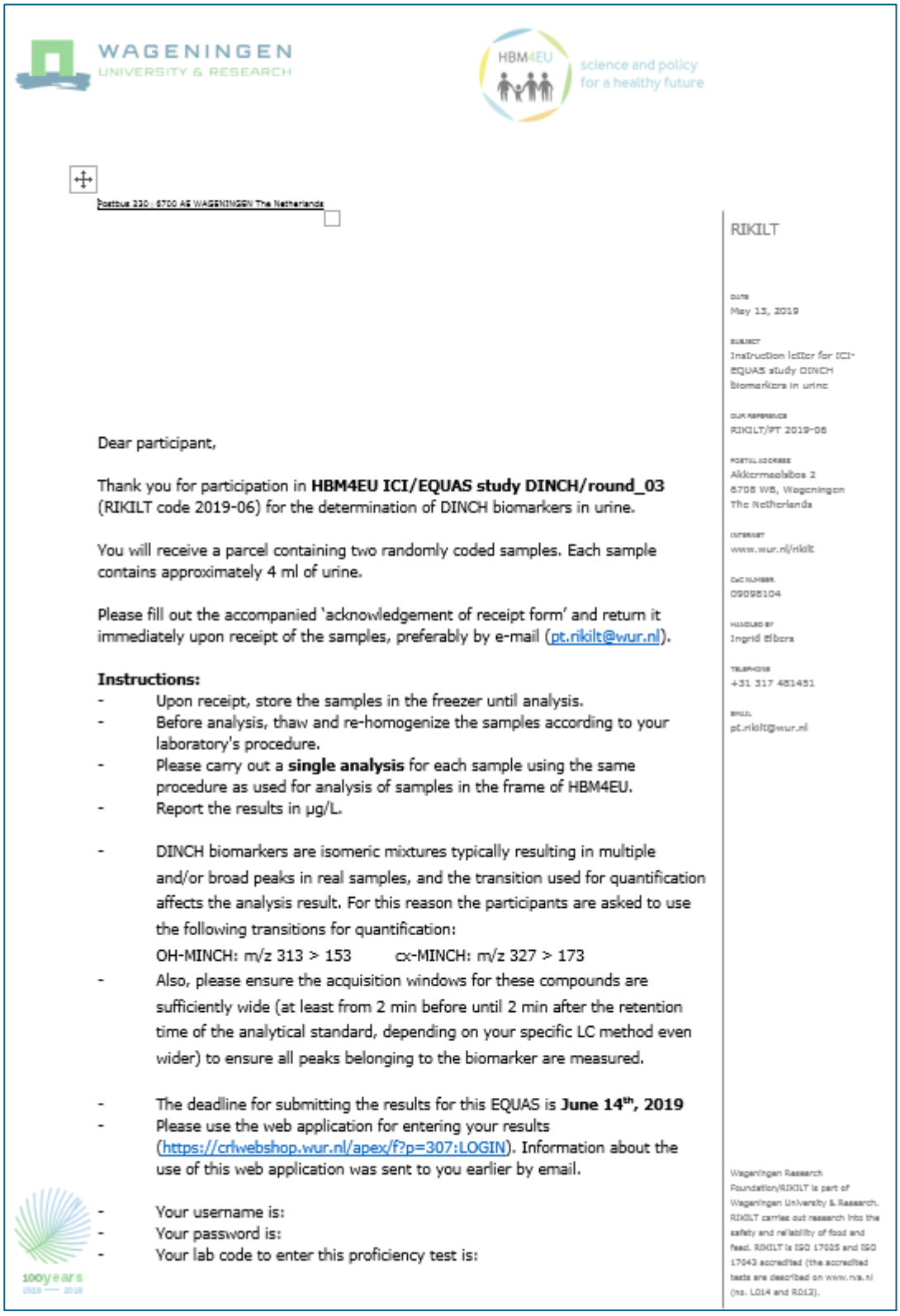
I hereby accept the conditions for participation as outlined in the letter accompanying this form.

Date / Signature:

Please sign a print of this document and e-mail a scan to pt.rikilt@wur.nl.
Please subscribe before 7th May, 2019

Ingrid Elbers (organiser proficiency test/ICI/EQUAS)
Hans Mol (scientific expert)
RIKILT - Wageningen University & Research
The Netherlands

Appendix 4. Copy of letter/instructions sent together with test samples



WAGENINGEN
UNIVERSITY & RESEARCH

HBM4EU science and policy for a healthy future

Postbus 230 | 6700 AE WAGENINGEN The Netherlands

RIKILT

DATE
May 13, 2019

SUBJECT
Instruction letter for ICI-EQUAS study DINCH Biomarkers in urine

OUR REFERENCE
RIKILT/PT 2019-06

POSTAL ADDRESS
Akkermaalsbos 2
6708 WB, Wageningen
The Netherlands

INTERNET
www.wur.nl/rikilt

CoC NUMBER
09098104

HANDLED BY
Ingrid Elbers

TELEPHONE
+31 317 481451

EMAIL
pt.rikilt@wur.nl

Dear participant,

Thank you for participation in **HBM4EU ICI/EQUAS study DINCH/round_03** (RIKILT code 2019-06) for the determination of DINCH biomarkers in urine.

You will receive a parcel containing two randomly coded samples. Each sample contains approximately 4 ml of urine.

Please fill out the accompanied 'acknowledgement of receipt form' and return it immediately upon receipt of the samples, preferably by e-mail (pt.rikilt@wur.nl).

Instructions:

- Upon receipt, store the samples in the freezer until analysis.
- Before analysis, thaw and re-homogenize the samples according to your laboratory's procedure.
- Please carry out a **single analysis** for each sample using the same procedure as used for analysis of samples in the frame of HBM4EU.
- Report the results in µg/L.
- DINCH biomarkers are isomeric mixtures typically resulting in multiple and/or broad peaks in real samples, and the transition used for quantification affects the analysis result. For this reason the participants are asked to use the following transitions for quantification:
  OH-MINCH: m/z 313 > 153 cx-MINCH: m/z 327 > 173
- Also, please ensure the acquisition windows for these compounds are sufficiently wide (at least from 2 min before until 2 min after the retention time of the analytical standard, depending on your specific LC method even wider) to ensure all peaks belonging to the biomarker are measured.
- The deadline for submitting the results for this EQUAS is **June 14th, 2019**
- Please use the web application for entering your results (https://crlwebshop.wur.nl/apex/f?p=307:LOGIN). Information about the use of this web application was sent to you earlier by email.
- Your username is:
- Your password is:
- Your lab code to enter this proficiency test is:

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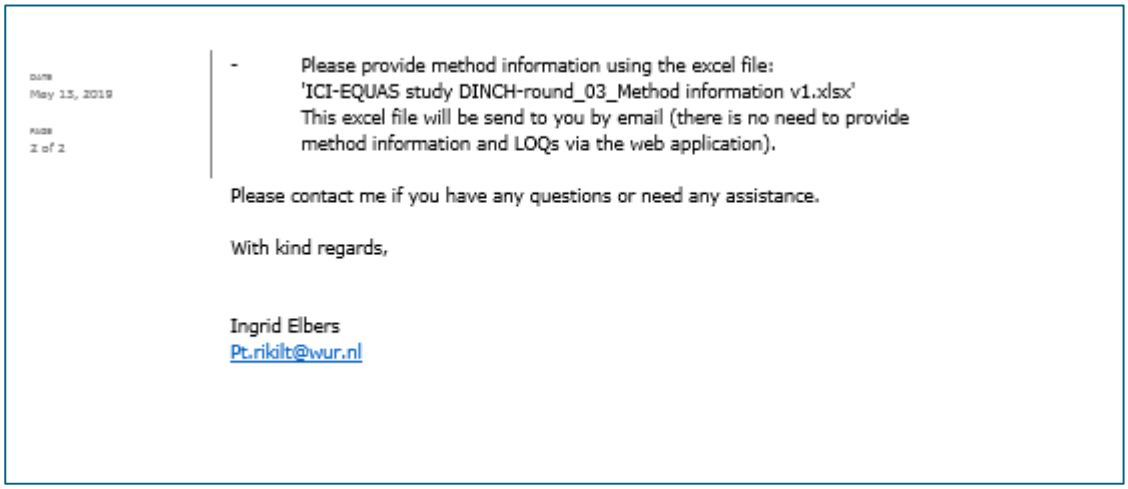
Wageningen Research Foundation/RIKILT is part of Wageningen University & Research. RIKILT carries out research into the safety and reliability of food and feed. RIKILT is ISO 17025 and ISO 17043 accredited (the accredited tests are described on www.rva.nl (no. L014 and R012).

Appendix 4. Copy of letter/instructions sent together with test samples (continued)



DATE
May 13, 2019

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- Please provide method information using the excel file:
  'ICI-EQUAS study DINCH-round_03_Method information v1.xlsx'
  This excel file will be send to you by email (there is no need to provide method information and LOQs via the web application).

Please contact me if you have any questions or need any assistance.

With kind regards,

Ingrid Elbers
Pt.rikilt@wur.nl

Appendix 5. Scope and LOQs as provided in the method information submitted by the laboratories.

	OH-MINCH	cx-MINCH	
Lab code	LOQ (ng/ml)		total
PT9572	0.4	0.5	2
PT9574	0.1	0.1	2
PT9575	0.1	0.1	2
PT9579	0.37	0.37	2
PT9581	0.2	0.2	2
PT9584	0.5	0.5	2
PT9585	0.2	nt	1
PT9586	1	0.5	2
PT9587	0.14	0.1	2
PT9588	0.2	0.2	2
PT9589	0.2	0.2	2
PT9604	0.05	0.05	2
total	12	11	

nt: not tested/not in the scope of the method

Appendix 6. Assigned values and Z-scores

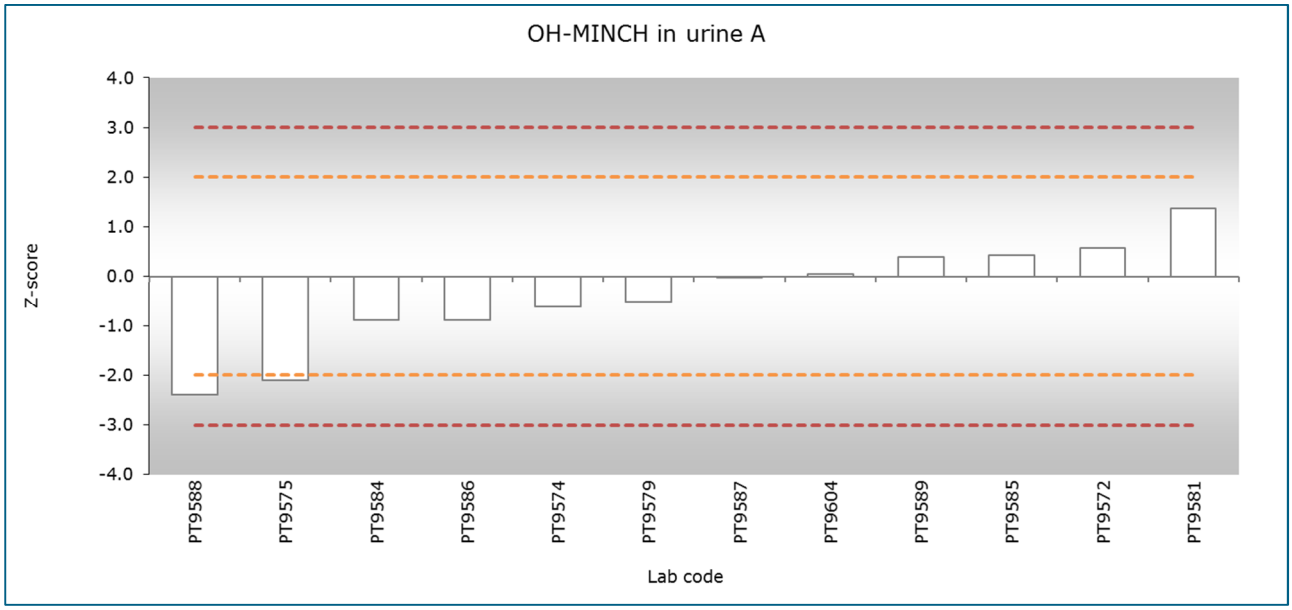
Control material	Test sample A		Test sample B		Test sample A		Test sample B	
Biomarker	**OH-MINCH**				**cx-MINCH**			
Conc. hom. test (ng/ml)	1.11		13.5		1.17		8.60	
Assigned value (ng/ml)	**1.09**		**13.0**		**1.09**		**8.3**	
Uncertainty	8.6%		2.0%		3.4%		6.2%	
Robust mean	0.953		10.7		u>>		5.04	
Study RSD_R	41%		19%		53%		15%	
Lab code	ng/ml	Z-score	ng/ml	Z-score	ng/ml	Z-score	ng/ml	Z-score
PT9572	1.25	0.6	12.8	0.0	1.05	-0.2	7.3	-0.5
PT9574	0.925	-0.6	12.6	-0.1	1.057	-0.1	9.00	0.3
PT9575	0.517	**-2.1**	7.28	-1.8	nt		nt	
PT9579	0.952	-0.5	8.978	-1.2	0.511	**-2.1**	4.554	-1.8
PT9581	1.47	1.4	12.02	-0.3	1.02	-0.3	7.62	-0.3
PT9584	0.85	-0.9	10.6	-0.7	0.6	-1.8	5.24	-1.5
PT9585	1.2114	0.4	11.6955	-0.4	nt		nt	
PT9586	0.85	-0.9	9.97	-0.9	0.90	-0.7	4.92	-1.6
PT9587	1.09	0.0	12.1	-0.3	0.88	-0.8	6.49	-0.9
PT9588	0.44	**-2.4**	9.78	-1.0	0.28	**-3.0**	4.32	-1.9
PT9589	1.20	0.4	13.3	0.1	1.40	1.1	4.45	-1.9
PT9604	1.11	0.0	13.5	0.2	1.17	0.3	8.60	0.1

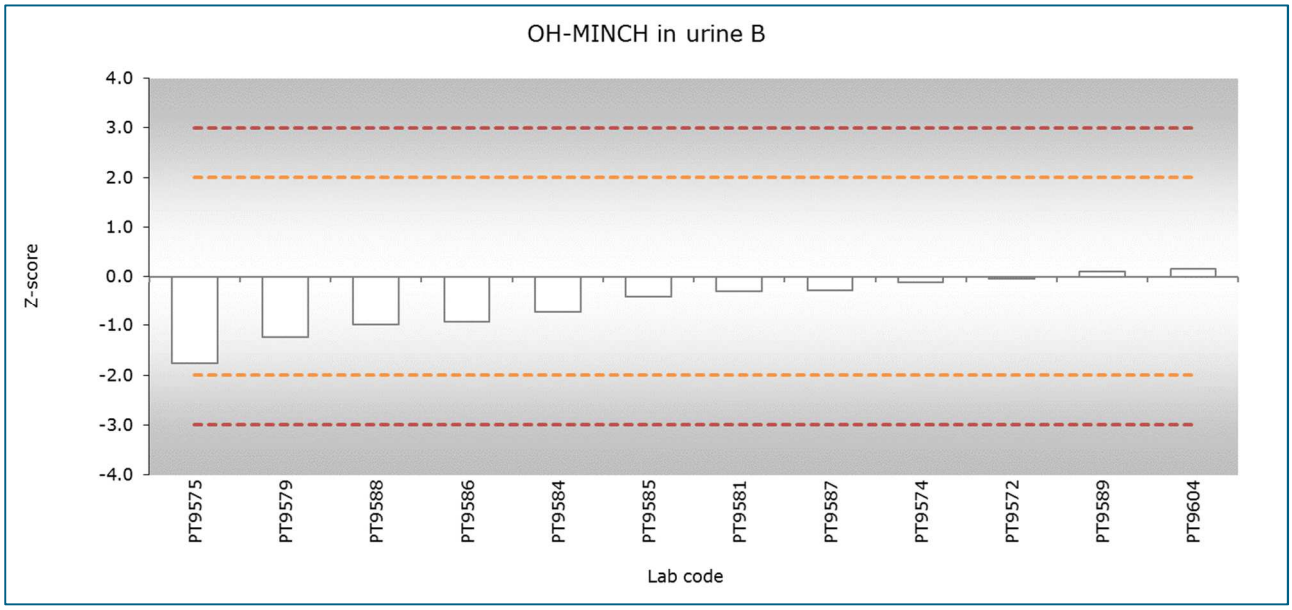
Assigned value is the expert-assigned value (mean of concentrations of three expert labs).

Study RSD_R is based on results from participants (excluding results from expert labs)

u>> = uncertainty of robust mean based on participants' results too large to calculate a meaningful robust mean

Appendix 7. Graphical representation of the Z-scores





Appendix 7. Graphical representation of the Z-scores (continued)