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Supporting Information

In-depth evaluation of automated non-contact reflectance-based hematocrit prediction of dried blood spots

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Supplementary Information Content

1. Experimental section

1.1 Selection of measurement conditions

The CAMAG® DBS-MS 500 HCT allows to adjust different instrumental parameters to determine the hematocrit (Hct) of a dried blood spot (DBS). Therefore, prior to the set-up of the calibration model, two instrumental parameters were optimized: (i) probe-to-card distance and (ii) integration time of one measurement. The number of 'sub-scans' per individual measurement was fixed and set at 16, as recommended by Luginbühl *et al.*.[1]

Optimization was performed using 24 venous left-over patient samples (Hct range 0.177 to 0.562 L/L). In a first step, the probe-to-card distance was varied from 3 to 12 mm, increasing with 1 mm per measurement and using a fixed integration time of 4500 µs. Also a probe-to-card distance of 6.4 mm with a fixed integration time of 4500 µs was evaluated, as these were the initial instrumental settings entered in the Chronos for CAMAG software. When oversaturation of the detector at a certain probe-to-card distance was observed (i.e. a background (BG) reflectance > 840), the integration time was reduced until the BG reflectance was below 840. Next, the optimal combination of probe-to-card distance and integration time was chosen based on the back-calculated Hct of the samples, using an initial linear calibration model. Based on the performance of the manual Hct prediction procedures[2-4], the acceptance limit was set at ±0.050 L/L difference from the reference value (determined using a hematology analyzer).

In addition to the aforementioned measurement conditions, the use of multiple measurements (further referred to as 'scans', with one 'scan' being the average of 16 'sub-scans') per DBS to determine the Hct was evaluated. First, as the reflectance of the DBS can be measured at different positions within the spot, the maximum x- and y-position of the probe to the center of the DBS where no BG reflectance is measured, was determined based on the mean diameter of a 25 µL DBS (n = 24; Hct range 0.177 to 0.562 L/L). In a next step, to evaluate the ideal number of 'scans' per DBS (n), a sample with a low (0.177 L/L), median (0.406 L/L) and high (0.562 L/L) Hct were 'scanned' at 21 different positions (Fig. S-11). For each sample, n was calculated using equation 1, in order to achieve a relative uncertainty of the mean normalized reflectance (i.e. $BG_{reflectance}/Hct_{reflectance}$) of maximum 5%.

$$\frac{CI_{(\alpha,n-1)}}{mean} \leq 0.05 \; with \; CI_{(\alpha,n-1)} = t_{(\alpha,n-1)} \cdot \frac{SD}{\sqrt{n}} \pm mean \quad (1)$$

The mean and standard deviation (SD) were calculated based on the data obtained from 21 'scans' and a two-tailed t-distribution and α = 0.05 were considered. The final number of 'scans' per DBS used in all further experiments was based on the mean of the result obtained for the three samples.

Finally, multiple scans at the center *vs.* determination of the Hct at different positions within the spot ('grid') were compared based on the data obtained during the set-up and validation of the calibration model.

2. Results and discussion

2.1 Selection of measurement conditions

When the probe-to-card distance was varied from 3 to 12 mm at a fixed integration time of 4500 µs, oversaturation of the detector was observed for a probe-to-card distance of 3, 4 and 5 mm. Therefore, the integration time was decreased to 1500, 2000 and 3000 µs, respectively (Table S-6). Furthermore, a probe-to-card distance of 6.4 mm in combination with an integration time of 4500 µs yielded the highest number of samples (92%) for which the back-calculated Hct values were within 0.050 L/L of the reference value (Table S-7). Hence, 6.4 mm and 4500 µs were selected as the optimal measurement conditions, which were in fact the initial instrumental parameters entered in the Chronos for CAMAG software. Since a non-weighted, linear regression equation to calculate the Hct was described in the CAMAG DBS-MS 500 HCT manual, Hct values were back-calculated based on an initial, non-validated linear calibration model.[5] In addition, a linear calibration model was used by Luginbühl *et al.* to predict the Hct of DBS in the context of a pharmacokinetic study of diclofenac.[1] Also here, the probe-to-card distance was evaluated, by varying the distance from 0.5 to 2.5 mm.[1] The authors concluded that the optimal probe-to-card distance was actually a range from 1.4 to 2.0 mm and continued with a distance of 1.8 mm for further experiments. However, we could not reproduce the recommended standard probe-to-card distance of 1.8 mm since the initial measurements were done using a vertical-positioned probe (prototype, Fig. S-12). Our system on the other hand, has a tilted probe with a minimal probe-to-card distance of 3 mm. More recently, Luginbühl *et al.* described the application of the automated Hct prediction method to correct for a Hct-dependent bias for the analysis of phosphatidylethanol, where a probe-to-card distance of 1.4 mm was applied to determine the Hct of the samples. Although this distance is within the previously validated range, it is inconsistent with the probe-to-card distance of 1.8 mm previously applied by these authors.[1, 6] Therefore, re-evaluation of the optimal probe-to-card distance was needed. Furthermore, since only a probe-to-card distance in a very low range (from 0.5 to 2.5 mm) was evaluated in these articles, back-calculated Hct values were only evaluated using a probe-to-card distance up till 7 mm. Additionally, when using a probe-to-card distance of 8 mm and higher, the beam of the excitation light appeared to be less focused on the DBS, with a less dense coverage of the DBS at the outer edge of the light beam, compared to when using a probe distance of 7 mm and lower (Fig. S-13).

The 'ideal' number of 'scans' per DBS for a sample with a low (0.177 L/L), median (0.406 L/L) and high (0.562 L/L) Hct were 5.9, 4.8 and 4.5, respectively, with a mean of 5 'scans' per DBS. Therefore, in all further experiments all samples were measured in fivefold either at the center of the DBS or using a grid. In addition, the mean diameter of a 25 µL DBS was approximately 8 mm, while the diameter of

90 the area of the light beam covering the DBS was approximately 4 mm. Consequently, to avoid
91 measurement of the BG when analyzing smaller DBS, the maximum x- and y-position of the probe from
92 the center of the spot used in the grid was set at 1.5 mm (Fig. S-4).

3. References

1. M. Luginbühl, Y. Fischer and S. Gaugler, *Journal of analytical toxicology*, 2020, **46**, 187-193.
2. S. Capiau, L. S. Wilk, M. C. Aalders and C. P. Stove, *Analytical chemistry*, 2016, **88**, 6538-6546.
3. S. Capiau, L. S. Wilk, P. M. M. De Kesel, M. C. G. Aalders and C. P. Stove, *Analytical chemistry*, 2018, **90**, 1795-1804.
4. L. Delahaye, L. Heughebaert, C. Luhr, S. Lambrecht and C. P. Stove, *Clinica chimica acta; international journal of clinical chemistry*, 2021, **523**, 239-246.
5. CAMAG, DBS-MS 500 HCT., https://dbs.camag.com/product/camag-dbs-ms-500-hct#downloads, (accessed 08/07/2022).
6. M. Luginbühl, F. Stoth, W. Weinmann and S. Gaugler, *Alcohol (Fayetteville, N.Y.)*, 2021, **94**, 17-23.

4. Supplementary Tables

Table S-1. Number of calibrators and QC samples (n) allocated to the different Hct cohorts: < 0.20, 0.20-0.25, 0.25-0.30, 0.30-0.35, 0.35-0.40, 0.40-0.45, 0.45-0.50 and > 0.50.

Hct cohort	< 0.20	0.20-0.25	0.25-0.30	0.30-0.35	0.35-0.40	0.40-0.45	0.45-0.50	> 0.50	Total
Number of calibrators (n)	11	14	11	12	12	13	11	11	95
Number of QCs (n)	6	5	5	5	5	5	5	6	42

Table S-2. Number of samples (%) for which the predicted Hct values were within ±0.050 L/L of the reference value (Sysmex) when applying a fivefold scan at the center of the spot or a 5-position grid (fivefold scan at different positions within the spot). For the QCs, the results obtained at Day 0 were used.

	Back-calculated calibrators (n = 95; RT) (%)	Back-calculated calibrators (n = 95; 60 °C) (%)	QC set 1 replicate 1 (n = 42) (%)	QC set 1 replicate 2 (n = 42) (%)	QC set 2 replicate 1 (n = 42) (%)	QC set 2 replicate 2 (n = 42) (%)
5 x center	82	82	88	90	79	79
5-position grid	88	87	90	90	93	88

Table S-3. Accuracy (bias, % and L/L) and precision (CV, %) based on the analysis of a second set of QC samples (n = 42, n = 5 per level, except for Hct levels < 0.20 and > 0.50 L/L, where n = 6 per level). Samples were measured twice per day on 4 different days.

Hct range	Intra-day precision	Total precision	Bias	
	CV (%)	CV (%)	%	L/L
< 0.20	0.8%	1.5%	6.8%	0.012
0.20-0.25	1.4%	1.5%	2.0%	0.005
0.25-0.30	1.3%	1.3%	1.4%	0.003
0.30-0.35	1.7%	1.7%	-1.6%	-0.004
0.35-0.40	3.6%	3.6%	2.4%	0.009
0.40-0.45	2.1%	2.1%	4.1%	0.017
0.45-0.50	1.2%	1.3%	1.8%	0.009
> 0.50	1.7%	2.6%	-7.0%	-0.036
Total (42)	1.9%	1.9%	1.2%	0.002

Table S-4. Accuracy (bias, % and L/L) and precision (CV, %) based on the analysis of a first set of QC samples using the aged calibration curve (n = 42, n = 5 per level, except for Hct-levels < 0.20 and > 0.50 L/L, where n = 6 per level). Samples were measured twice per day on 4 different days.

Hct range	Intra-day precision	Total precision	Bias	
	CV (%)	CV (%)	%	L/L
< 0.20	1.2%	1.8%	2.9%	0.005
0.20-0.25	1.7%	2.0%	1.4%	0.003
0.25-0.30	1.6%	2.0%	-5.2%	-0.015
0.30-0.35	1.5%	2.0%	-5.0%	-0.015
0.35-0.40	1.5%	1.7%	-5.6%	-0.019
0.40-0.45	1.4%	1.9%	-4.6%	-0.020
0.45-0.50	1.1%	1.9%	0.4%	0.002
> 0.50	2.2%	2.6%	-5.4%	-0.028
Total (42)	1.6%	2.0%	-2.6%	-0.011

Table S-5. Accuracy (bias, % and L/L) and precision (CV, %) based on the analysis of a second set of QC samples using the aged calibration curve (n = 42, n = 5 per level, except for Hct-levels < 0.20 and > 0.50 L/L, where n = 6 per level). Samples were measured twice per day and on 4 different days.

Hct range	Intra-day precision	Total precision	Bias	
	CV (%)	CV (%)	%	L/L
< 0.20	0.9%	1.6%	-1.0%	-0.002
0.20-0.25	1.5%	1.6%	-4.4%	-0.010
0.25-0.30	1.3%	1.3%	-4.2%	-0.012
0.30-0.35	2.0%	2.0%	-6.5%	-0.020
0.35-0.40	1.6%	1.8%	-2.9%	-0.011
0.40-0.45	2.1%	2.1%	-0.7%	-0.003
0.45-0.50	1.2%	1.2%	-3.0%	-0.014
> 0.50	1.6%	2.5%	-11.3%	-0.058
Total (42)	1.6%	1.8%	-4.4%	-0.016

Table S-6. Combination of probe-to-card distance (mm) and integration time (µs) where no oversaturation of the detector was observed.

Probe-to-card distance (mm)	Integration time (µs)
3	1500
4	2000
5	3000
6	4500
6.4	4500
7	4500
8	4500
9	4500
10	4500
11	4500
12	4500

Table S-7. Number of samples (n = 24, %) using a certain probe-to-card distance (mm) and integration time (µs) for which the difference between the back-calculated Hct and the reference (Sysmex) was within ±0.050 L/L. Back-calculated Hct levels were based on an initial, linear calibration model.

Probe-to-card distance (mm)	Integration time (µs)	Back-calculated Hct within ± 0.050 L/L of the reference (%)
3	1500	67
4	2000	75
5	3000	62
6	4500	87
6.4	4500	92
7	4500	87

5. Supplementary Figures

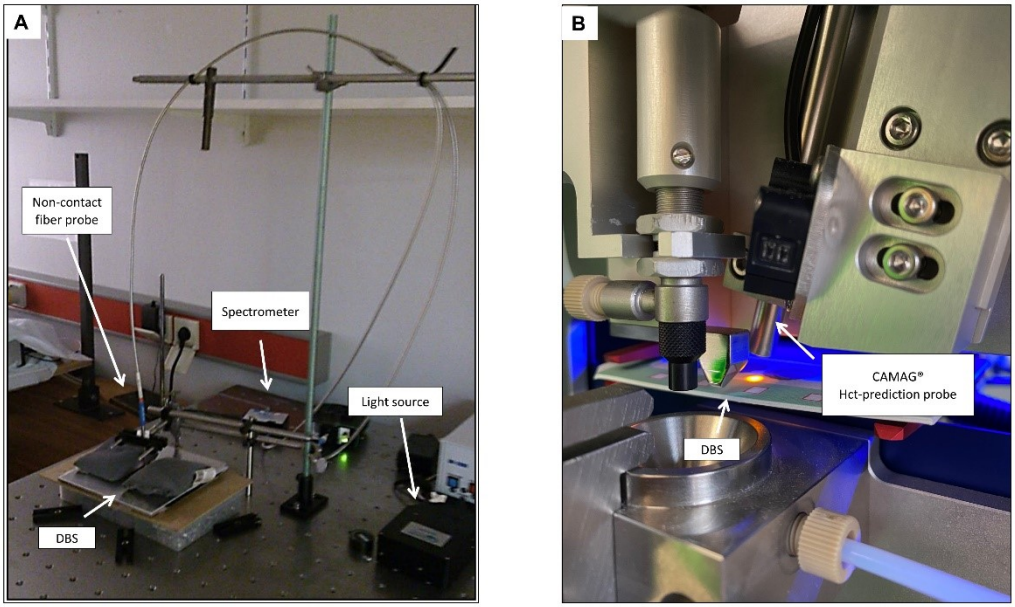
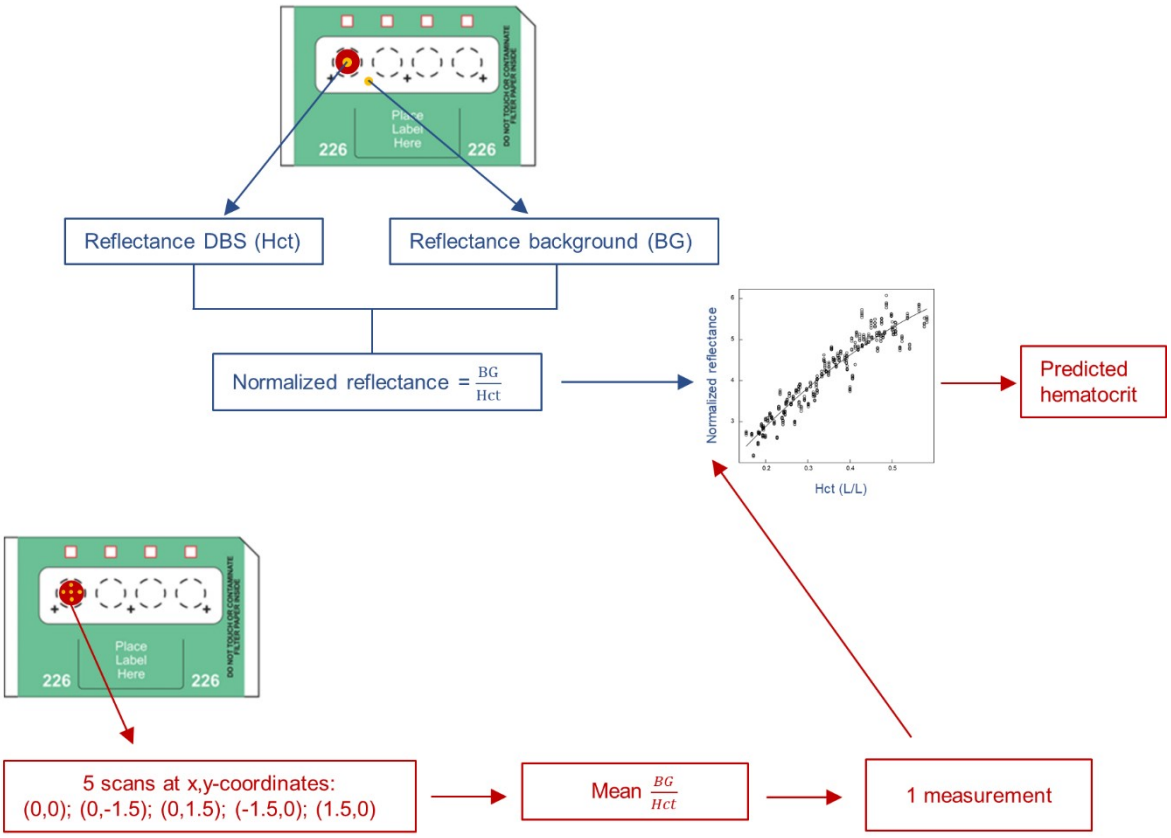


Fig. S-1. Depicted are a picture of (A) the initial, manual set-up to predict the Hct via UV-Vis spectroscopy developed and described by Capiau *et al*.[2] and (B) the automated Hct prediction module installed into the CAMAG DBS-MS 500 HCT system. The different parts required for the analysis are indicated.

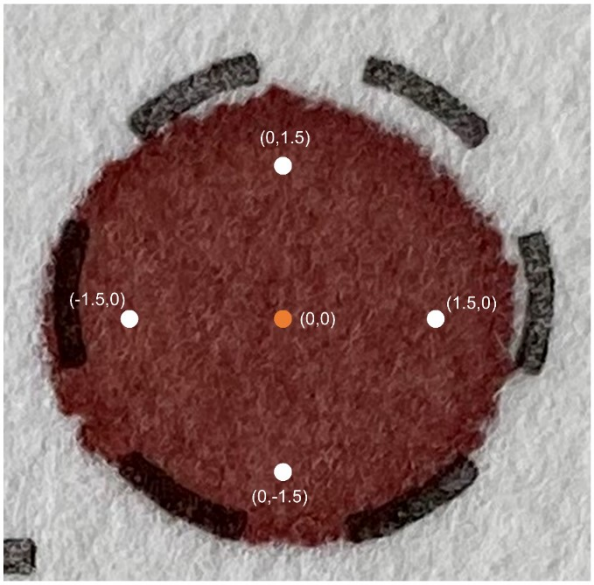


141

142 Fig. S-2. Schematic overview of how the spectral data is processed to obtain a Hct value.

143

144 Fig. S-3. Depicted is a picture of four DBS calibration samples which were stored for 24 h (A) at room
145 temperature (RT) and (B) at 60 °C, the latter to mimic ageing of the DBS. Note the difference in color –
146 the samples stored at 60 °C being more brown.



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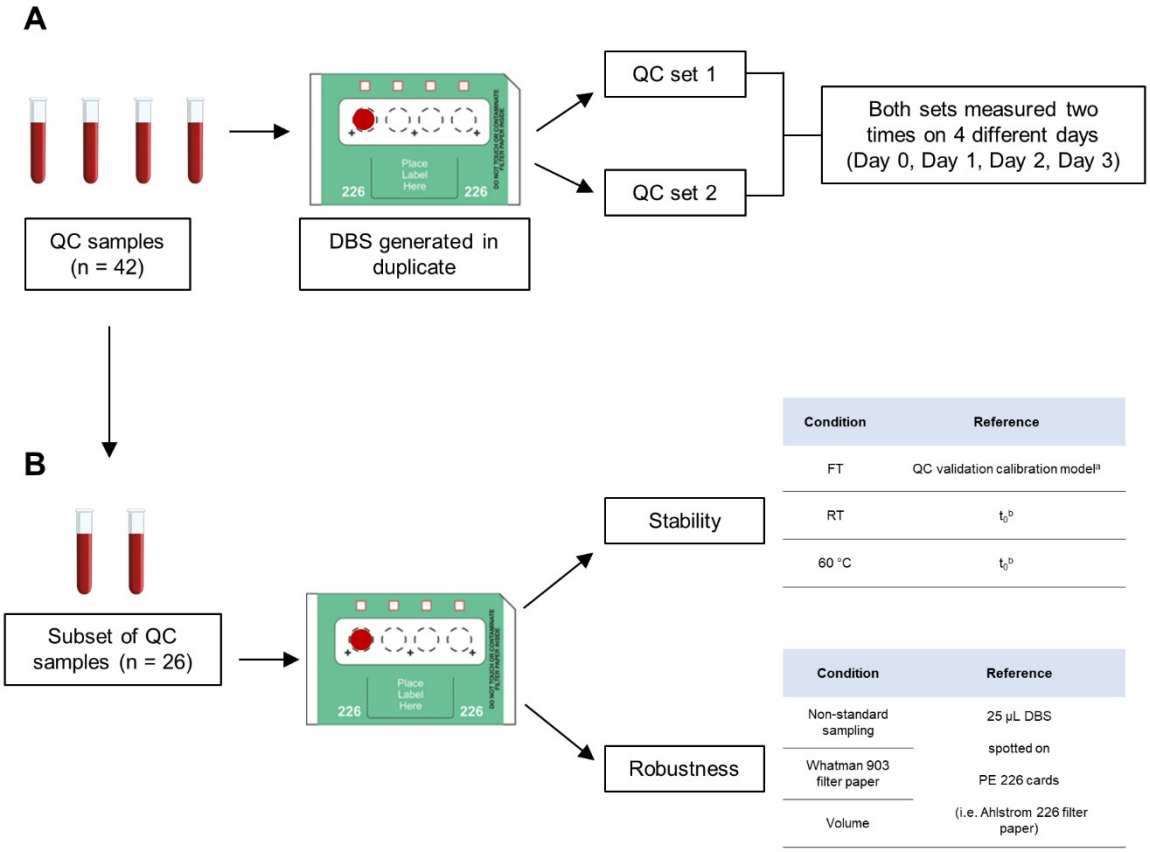
148 Fig. S-4. Depicted is a 25 µL DBS with the 5-position grid indicated by its x,y-coordinates (mm). Both
149 calibrators and QCs were scanned in fivefold at the center of the spot (orange dot) and at five different
150 positions (orange and white dots).

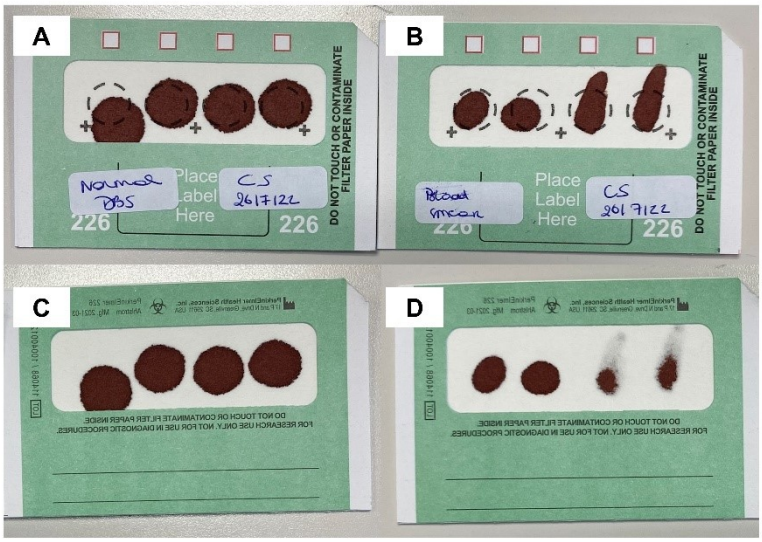


151

152 Fig. S-5. Schematic overview of (A) the analysis of the QC samples and (B) the analysis of the samples
153 (i.e. subset of the QC samples) used for evaluation of stability and robustness and how the data analysis
154 was performed.

155 [a]Samples were compared to a different DBS (inter-spot comparison).

156 [b]Samples were compared to the very same DBS (intra-spot comparison).



157

158 Fig. S-6. Depicted is a photograph of the front and back (the latter mirrored) of capillary DBS with a
159 round, normal shape (A & C, respectively) and an atypical shape ('blood smear') (in B & D (3rd and 4th
160 DBS), respectively).

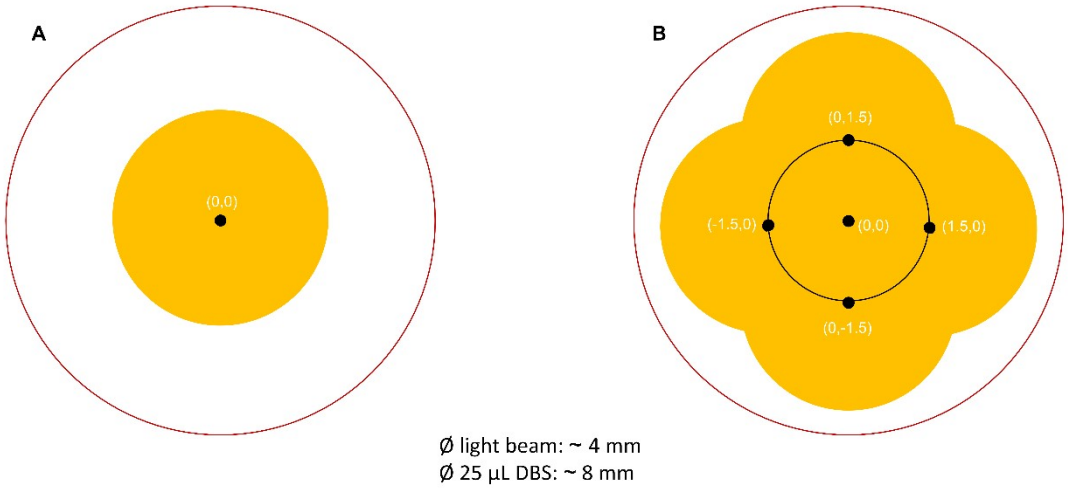
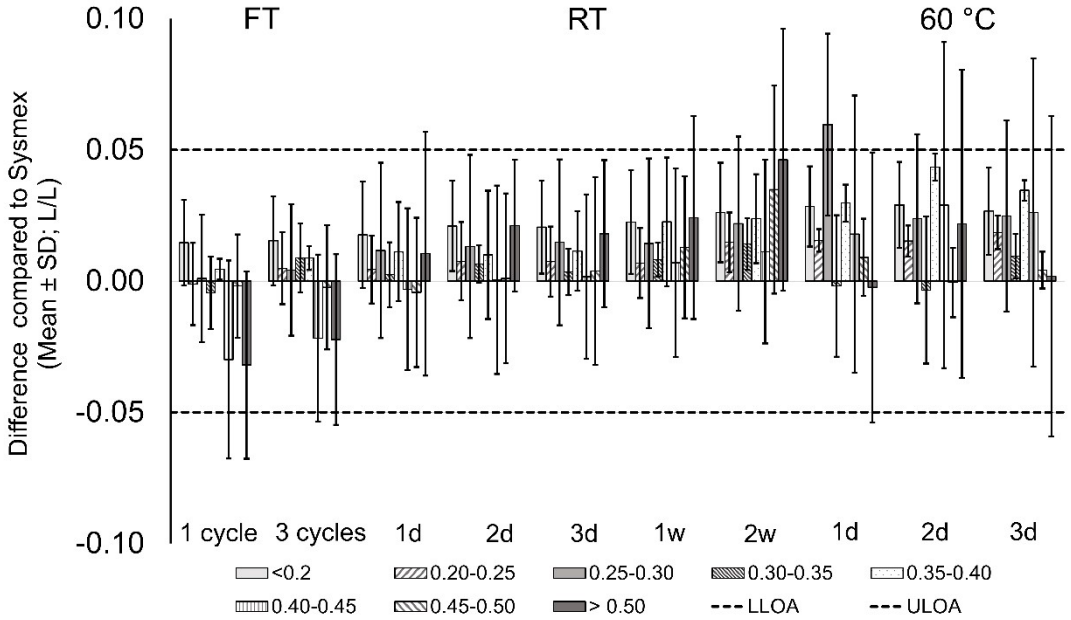


Fig. S-7. Approximate area of the DBS measured by the spectrophotometer (A) when no grid is used and (B) when a grid is used. The x,y-coordinates (mm) from the center are indicated in the Figures. The diameter (Ø) of the light beam is approximately 4 mm and the diameter of a 25 µL DBS (used as reference) is approximately 8 mm.



166

167 Fig. S-8. Stability results after one and three freeze-thaw (FT) cycles, storage at room temperature (RT)
168 and storage at 60 °C. The mean difference ± standard deviation (SD) per Hct level (L/L) compared to
169 the reference value (Sysmex) is shown (n = 3 per level, except for Hct levels < 0.20 and > 0.50 L/L,
170 where n = 4 per level). The dashed line indicates the acceptance limit of ±0.050 L/L difference.

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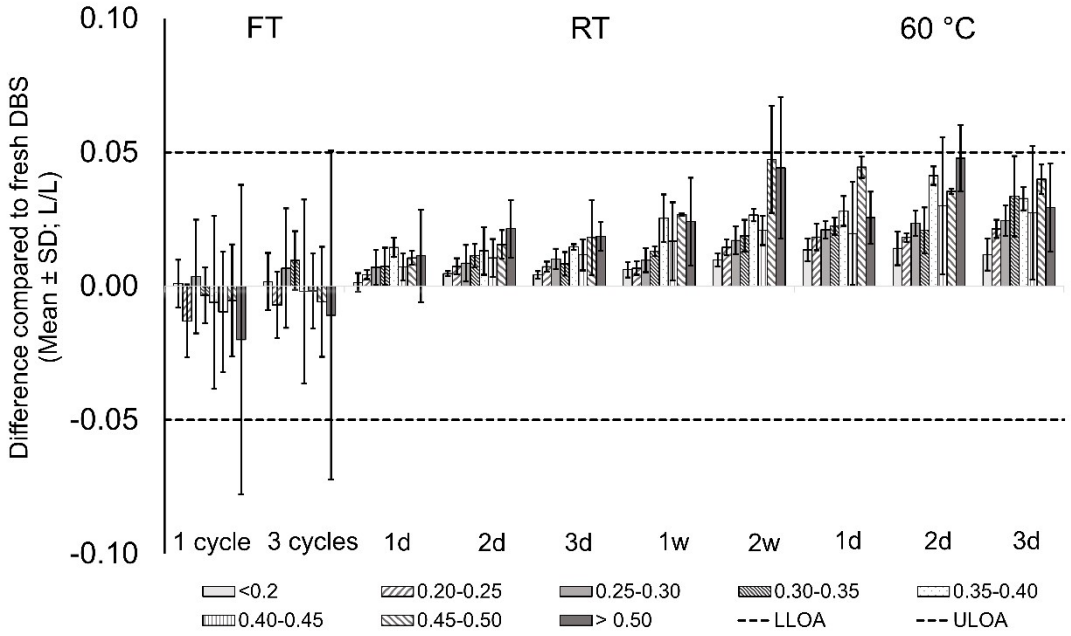
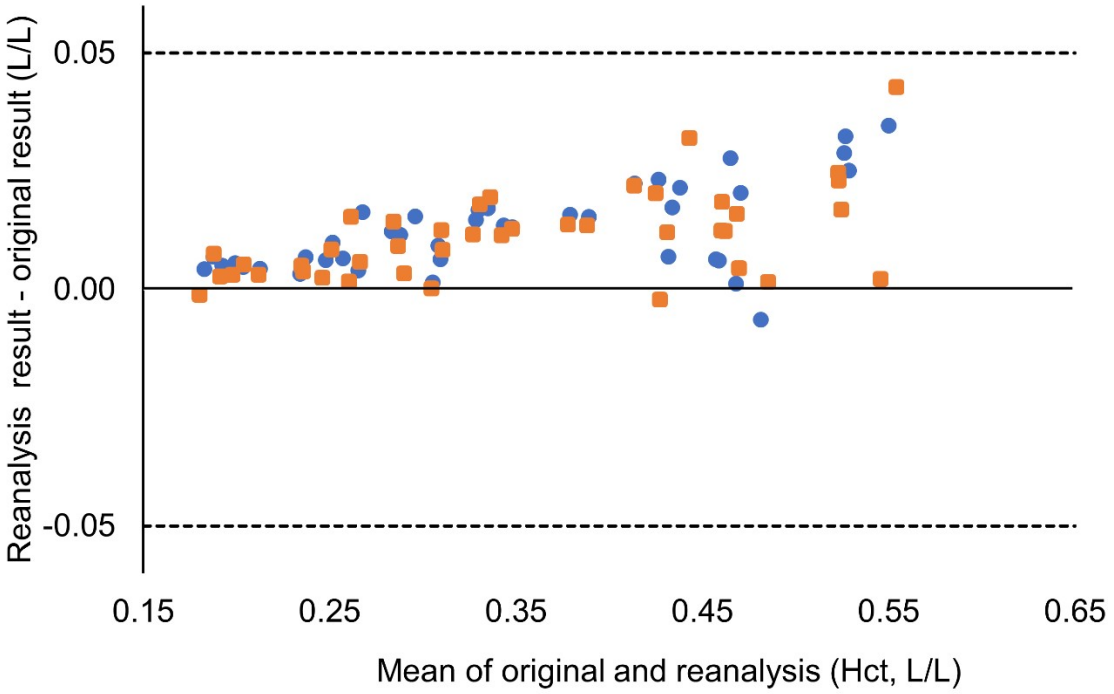


172

173 Fig. S-9. Stability results after one and three freeze-thaw (FT) cycles, storage at room temperature (RT)
174 and storage at 60 °C. Results were obtained using the aged calibration curve (i.e. stored for one day at
175 60 °C). The mean difference in Hct prediction ± standard deviation (SD) per Hct-level (L/L) compared
176 to fresh DBS is shown (n = 3 per level, except for Hct-levels < 0.20 and > 0.50 L/L, where n = 4 per level).
177 The dashed line indicates the acceptance limits of ±0.050 L/L difference.



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179 Fig. S-10. Incurred sample reanalysis (n = 42x2). The Hct predictions on Day 0 (DBS 1) were compared
180 to (i) the results of the same spot (DBS 1) on Day 3 (blue circles) and (ii) the results of the replicate spot
181 (DBS 2) on Day 3 (orange squares). The dashed line indicates the acceptance limits of ±0.050 L/L
182 difference, which was met by all samples.

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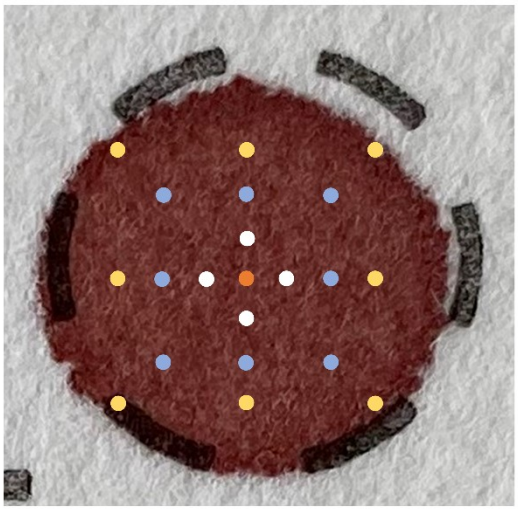

Fig. S-11. Depicted is a DBS where the 21 different positions where the sample was ‘scanned’ to evaluate the ideal number of ‘scans’ per DBS are indicated. The center of the spot (0,0) is indicated by the orange dot. The x,y-coordinates were set at 0.5 mm (white dots), 1.0 mm (blue dots) and 1.5 mm (yellow dots).

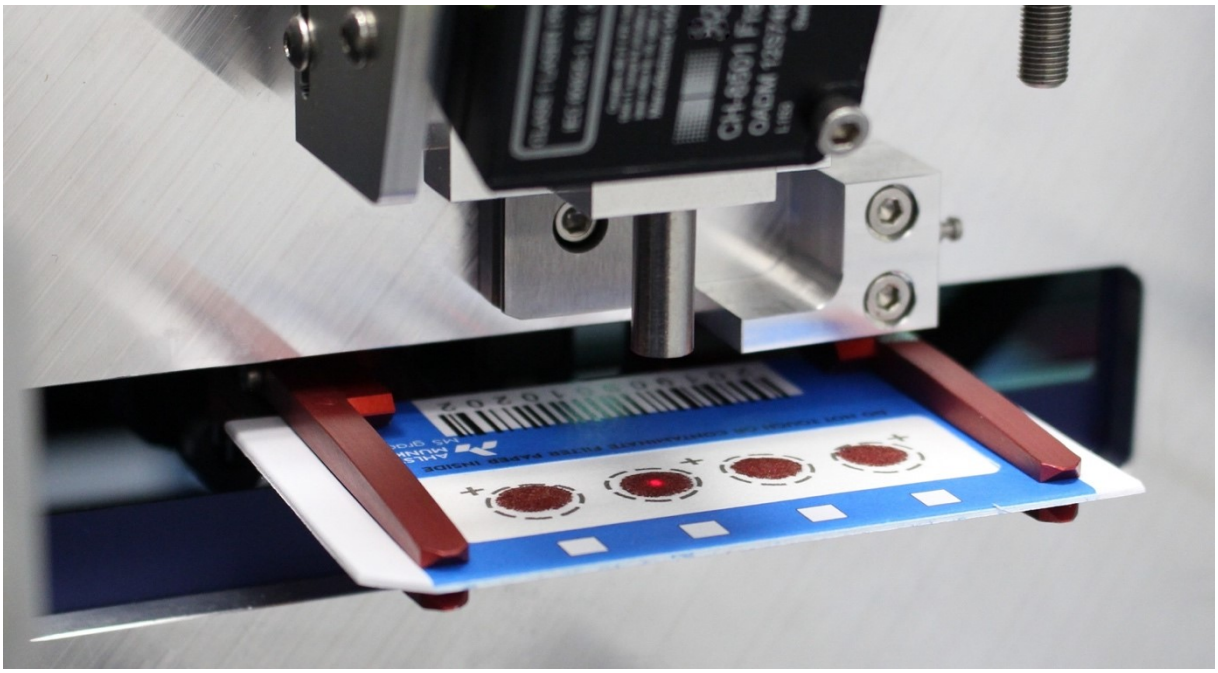

Fig. S-12. Depicted is a picture of the prototype Hct prediction module (vertical probe).

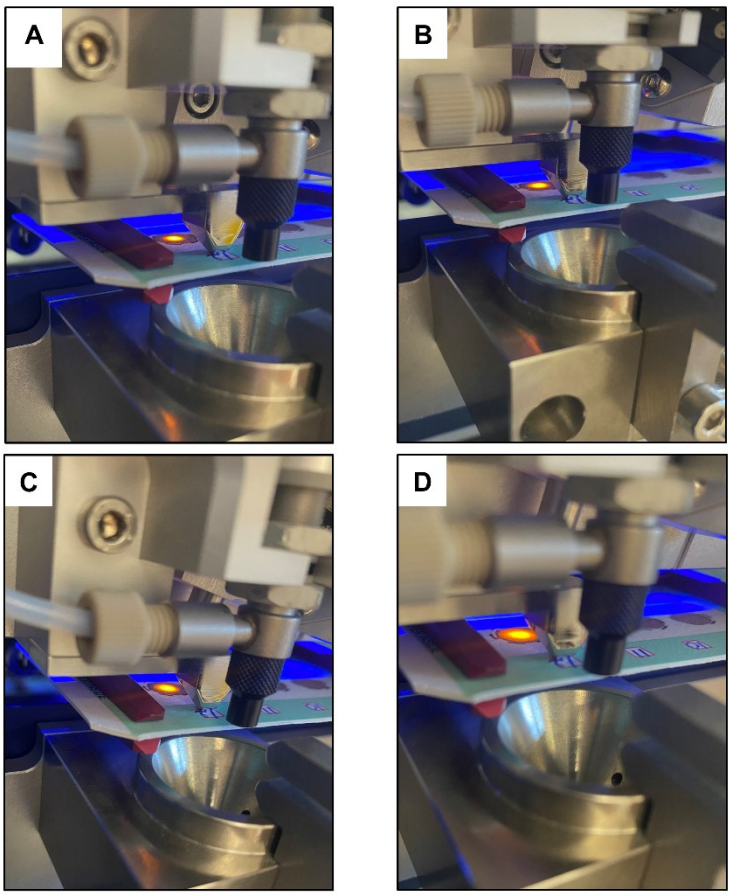

Fig. S-13. Depicted are pictures of the automated Hct-prediction module, analyzing a DBS using different probe-to-card distances: (A) 6.4 mm; (B) 7 mm; (C) 8 mm and (D) 12 mm.