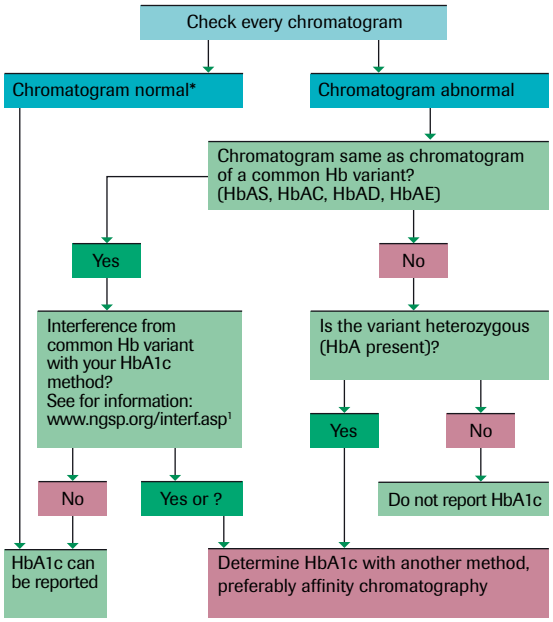
Hemoglobin variant study
Turbidimetry versus HPLC

Lenters-Westra, E., Siebelder, C., Slingerland, R.J., Weykamp, C.W.
IFCC/NGSP Reference Laboratory (European Reference Laboratory, NL)

Introduction

Ideally, a patient who has been diagnosed with diabetes would also be screened for hemoglobinopathies and thalassemia. This information could then be used to choose the right method to reliably measure hemoglobin A1c (HbA1c) and also for genetic counseling to avoid Hb major in newborns. Unfortunately, however, this is still not common clinical practice.

The most common remark from technologists using cation exchange high performance liquid chromatography (HPLC) to measure HbA1c, as opposed to immunoassays, is that they want to know if there is a variant present or not. In most cases, a variant will result in an abnormal chromatogram, whereas variant identification is not possible with immunoassays. However, this means every chromatogram should be checked manually for abnormalities prior to reporting the HbA1c result. While this process may enable the identification of potential interference from some Hb variants, more than 1,175* different variants exist. Furthermore, some Hb variants co-elute with HbA generating a normal chromatogram. In these cases, the HbA1c result would be artificially low or high, depending on which Hb variant is present. Figure 1 shows the complexity of HbA1c measurements using cation exchange HPLC. If this flow chart is followed, cation exchange HPLC is a method for determining HbA1c in the presence of Hb variants.



**A normal chromatogram does not guarantee that there is no Hb variant present. If a variant is present the HbA1c value will be falsely low or high, depending on the variant*

Fig. 1: Complexity of HbA1c measurement with cation exchange HPLC in the presence of Hb variants

When using an immunoassay, technologists are often concerned that the test gives no indication of whether or not a variant is present. However, an immunoassay does not need to identify individual Hb variants because all forms of Hb are included for the HbA1c calculation. As long as there are no mutations in the epitope for the antibody used in the assay the HbA1c measurement is not affected.

**** See Addendum II on page 13 for updated statistics***

For the vast majority of the more than 1,175* Hb variants identified to date,[2] the mutation is not in the first four amino acids of the Hb protein and therefore does not interfere with the Tina-quant® HbA1c Gen.2 and Gen.3 assays from Roche; the mutation is in this region for only a very small number of variants (17/1,175*).[2]

Furthermore, it is important to note that the prevalence of these 17* Hb variants is very low, and often they only occur in certain individuals (e.g. Hb Okayama in Japanese men) or in a few families, e.g. (Hb Graz in Austria) globally. Figure 2 shows the complexity of HbA1c measurement with an immunoassay and Table 1 lists some of the rare Hb variants that can interfere with the Tina-quant® HbA1c Gen.2 and Gen.3 assays.

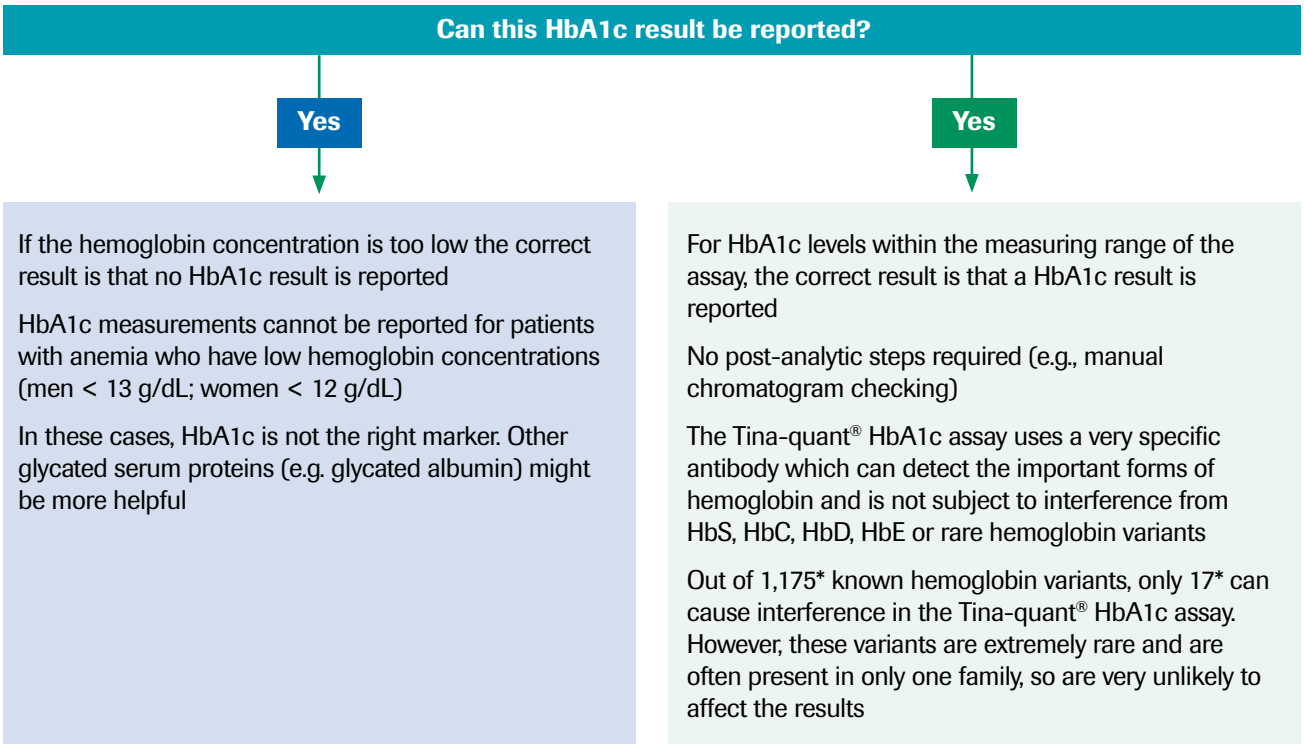


Fig. 2: Complexity of HbA1c measurement with an immunoassay in the presence of hemoglobin variants

Hb variant	Substitution of amino acid
Hb Raleigh	β1 Val → Ala
Hb Niigata	β1 Val → Leu
Hb Deer Lodge	β2 His → Arg
Hb Okayama	β2 His → Gln
HHb Graz	β2 His → Leu
Hb Agrigente	β2 His → Pro
Hb Fukuota	β2 His → Tyr

Table 1: Hemoglobin variants that can interfere with the Tina-quant® HbA1c Gen.2 and Gen.3 assays and potentially also with cation exchange HPLC[2]

Study design

The study objective was to investigate the potential interference of different Hb variants with different analytical methods for measuring HbA1c.

Samples were analyzed at two sites using the following methods:

Site 1: IFCC/NGSP Reference Laboratory (European Reference Laboratory, Isala, Zwolle, NL)

- Tina-quant® HbA1c Gen.2 on the COBAS INTEGRA® 800 analyzer; IFCC and NGSP certified immunoassay (Roche)
- Trinity Ultra,[2] IFCC and NGSP certified affinity chromatography HPLC (Trinity Biotech)
- Tosoh G8; IFCC certified cation-exchange HPLC (Tosoh Bioscience).

*See Addendum II on page 13 for updated statistics

Site 2: IFCC/NGSP Reference Laboratory (European Reference Laboratory, Queen Beatrix Hospital, Winterswijk, NL)

- Tina-quant® HbA1c Gen.2 on the **cobas c** 501 module; immunoassay (Roche)
- Tina-quant® HbA1c Gen.3 on the **cobas c** 501 module; immunoassay (Roche)
- Menarini HA-8180V; IFCC and NGSP certified cation exchange HPLC (A. Menarini Diagnostics).**

The IFCC reference system is currently the only valid method for standardizing HbA1c measurements.[3] All instruments and methods were calibrated using the IFCC secondary reference material (batch Berlin). The IFCC calibrators were run as samples and IFCC offline calibrated values calculated retrospectively. The final data evaluation to determine any potential interference of the variant was based on the IFCC offline calibrated results for all methods.

As the majority of Hb variants are considered not to interfere with Trinity Ultra[2] affinity chromatography, this method served as a comparator and all samples were analyzed in duplicate using this method.

For the other methods investigated, if 10 or more samples of one variant type were available, samples were measured individually, otherwise samples were tested in duplicate. All samples were investigated over 5 runs on 5 different days of about equal length.

Sample material consisted of whole blood from single donors. HbF samples comprised a mixture of blood from adults and neonates.

For variants Hb Volga and HbSS, no results could be reported. In the case of HbF, the mean value of the Menarini HA-8180V and the Tosoh G8 was taken as the reference method.

Significant differences between each method and the comparator, and recoveries between normal HbAA samples and samples containing variant Hb were calculated using a Deming regression at a target value of 42 mmol/mol and 75 mmol/mol, respectively. The maximum expected deviation was ~ 5 % and a deviation > 10 % was considered to be significant.

The results were also plotted in a graph and if the results of samples with Hb variants fell within the dispersion of the normal HbAA samples the variant was considered not to interfere with the method.

Results and discussion

Results using Tina-quant® HbA1c assay from Roche

The study shows that the Hb variants HbAS, HbAC, HbAD, HbAE, HbA2, HbAJ, HbAG and the rare variants do not interfere with the Tina-quant® HbA1c Gen.2 and Gen.3 assays run on the **cobas c** 501 module or the Tina-quant® HbA1c Gen.2 assay run on the COBAS INTEGRA® 800 analyzer (Table 2, Figures 3–5, 8–10, 13–15, 17–19 and 21–23). Only HbF > 8 % and the Hb Okayama variant were found to interfere with the Tina-quant® HbA1c Gen.2 assay (Figures 26–28).

The effect of Hb Okayama can be explained by the fact that the antibodies used in these assays target the four amino acids and the glucose at the N-terminal end of the hemoglobin β-globin chain. In the case of Hb Okayama, there is a substitution of the second amino acid (β-2 His → Gln) and therefore this variant will not be recognized by the antibodies in the assay. HbF does not glycate as fast as HbAA, but is included in the measurement of total Hb, which explains why the HbA1c result is falsely low. It is very difficult to get samples from patients with different HbF and HbA1c values and for this reason the HbF samples tested comprised mixtures of blood from adults and neonates. The literature confirms that most of the common HbA1c methods are affected when HbF is > 15 %.[4,5] In these studies samples from adults were used instead of mixtures of blood from adults and neonates and this could account for the fact that the current study observed interference at HbF > 8 % rather than HbF > 15 %.

Results using Menarini HA-8180V

The Menarini HA-8180V did not produce a HbA1c result for samples containing HbAD, HbAE, HbAJ, HbAG and most of the rare variants. However, reporting no result in the case of an abnormal chromatogram, where the Hb variant is not completely separated from the A0 peak, is the correct outcome. The Menarini HA-8180V did produce a result for samples containing HbAS and HbAC and elevated A2. The HbS and HbC peaks are completely separated from the A0 peak in the chromatogram and similarly the HbS1c and HbC1c peak are completely separated from the HbA1c peak. The software can therefore accurately subtract the area of the variant peak from the total area and calculate the HbA1c value correctly and these variants do not cause interference (Figures 6 and 11). All investigated variants except Hb Indonesia and Hb Hopkins-2 gave abnormal chromatograms with the Menarini HA-8180V and, in most cases, gave no HbA1c

*** Not available in the U.S.*

result (Table 2). Using this HPLC technique, the HbF peak is separated from the total area and therefore a true HbA1c result is obtained.

Results using Tosoh G8

Table 2 and Figures 7, 12, 16, 20 and 25 show that the Tosoh G8 produced a result for samples containing HbAS, HbAC, HbAD, HbAE, elevated A2, HbAG and HbAJ. However, interference due to HbAE and HbAJ was observed, with false low values obtained in samples containing these two variants (Figures 20 and 25). Figure 7 shows lower results obtained with the Tosoh G8 when samples contained the variant HbAS and the deviation from the HbAA samples was borderline (9.5 % deviation at 42 mmol/mol and 8.5 % at 75 mmol/mol).

See Addendum III on page 14 for NGSP conversion rate

cobas c 501 module with Tina-quant® HbA1c Gen.2 assay

	Linear Regression	Deming regression	R^2	42 mmol/mol	Significant difference	75 mmol/mol	Significant difference
AA	y = 1.07x - 3.67	y = 1.09x - 4.42	0.978	41		77	
AS	y = 1.04x - 2.91	y = 1.05x - 3.26	0.987	41	No	75	No
AC	y = 1.02x - 1.29	y = 1.03x - 1.64	0.985	42	No	76	No
AD	y = 0.96x + 0.37	y = 0.97x - 0.05	0.983	41	No	72	No
AE	y = 0.99x - 0.04	y = 1.00x - 0.44	0.958	42	No	74	No
HbF					> HbF 8 %		
A2					No		
Rare variants					Hb Okayama		

cobas c 501 module with Tina-quant® HbA1c Gen.3 assay

	Linear Regression	Deming regression	R^2	42 mmol/mol	Significant difference	75 mmol/mol	Significant difference
AA	y = 1.08x - 3.01	y = 1.09x - 3.70	0.980	42		78	
AS	y = 1.10x - 4.38	y = 1.10x - 4.68	0.990	42	No	78	No
AC	y = 1.01x - 0.77	y = 1.03x - 1.59	0.965	42	No	76	No
AD	y = 0.97x + 1.99	y = 0.98x + 1.41	0.976	43	No	75	No
AE	y = 1.08x - 2.89	y = 1.10x - 3.78	0.971	42	No	78	No
HbF					> HbF 8 %		
A2					No		
Rare variants					Hb Okayama		

COBAS INTEGRA® 800 analyzer with Tina-quant® HbA1c Gen.2

	Linear Regression	Deming regression	R^2	42 mmol/mol	Significant difference	75 mmol/mol	Significant difference
AA	y = 1.08x - 4.28	y = 1.08x - 4.56	0.992	41		77	
AS	y = 1.07x - 4.40	y = 1.08x - 4.72	0.989	41	No	76	No
AC	y = 1.00x - 0.72	y = 1.01x - 1.07	0.980	41	No	75	No
AD	y = 0.98x – 0.52	y = 0.99x - 0.82	0.988	41	No	73	No
AE	y = 0.99x - 0.47	y = 1.00x - 0.89	0.984	41	No	74	No
HbF					> HbF 8 %		
A2					No		
Rare variants					Hb Okayama		

However, it should be noted that the Deming regression lines calculated for the common variants were based on 20 samples compared with 50 samples for the normal HbAA and that the distribution of HbA1c over the clinically important range for HbA1c samples containing the common variants (HbAS, HbAC, HbAD and HbAE) was not always optimal. It is debatable, therefore, whether the method for calculating the deviation from normal samples is correct, because Figure 7 clearly shows a difference from normal HbAA samples. This phenomenon of lower results with HbAS variants using the Tosoh G7/G8 has been observed in previous studies (not published) and, as yet, there is no explanation given that the chromatograms show that the S0 peak is completely separated from the A0 peak, and hence a correct result would be expected. All investigated variants except Hb Indonesia and Hb Hopkins-2 gave abnormal chromatograms with the Tosoh G8 and, in most cases, gave no HbA1c result (Table 2). Using this HPLC technique, the HbF peak is separated from the total area and therefore a true HbA1c result is obtained.

Conclusion

The perfect method for measuring HbA1c still does not exist and which method is chosen will depend on the laboratory's priorities. Some will choose to use an immunoassay to obtain a fast and reliable HbA1c result regardless of the presence or absence of a Hb variant. Other laboratories, however, will want to know if a variant is present and opt for cation exchange HPLC. Hence, the advantages/ disadvantages of the different HbA1c methods will be viewed differently by different laboratories.

See Addendum III on page 14 for NGSP conversion rate

Menarini HA-8180V

	Linear Regression	Deming regression	R^2	42 mmol/mol	Significant difference	75 mmol/mol	Significant difference
AA	y = 1.03x -1.54	y = 1.03x - 1.90	0.989	41		76	
AS	y = 1.06x - 5.91	y = 1.07x - 6.31	0.989	39	No	74	No
AC	y = 1.02x - 0.46	y = 1.03x - 1.08	0.973	42	No	76	No
AD					No result		
AE					No result		
HbF							
A2					No		
Rare variants					All investigated rare variants except for Hb Indonesia and Hb Hopkins-2		

Tosoh G8

	Linear Regression	Deming regression	R^2	42 mmol/mol	Significant difference	75 mmol/mol	Significant difference
AA	y = 1.02x - 0.37	y = 1.02x - 0.67	0.990	42		76	
AS	y = 0.95x - 1.60	y = 0.96x - 1.97	0.984	38	No	70	No
AC	y = 1.02x - 1.95	y = 1.04x - 2.91	0.960	41	No	75	No
AD	y = 0.83x + 8.41	y = 0.85x + 7.19	0.935	43	No	71	No
AE	y = 0.69x + 0.80	y = 0.70x + 0.29	0.957	30	Yes	53	Yes
HbF							
A2					No		
Rare variants					All investigated rare variants except for Hb Indonesia and Hb Hopkins-2		

Table 2: Medical decision points calculated with Deming regression lines. A deviation > 10 % compared with HbAA samples was considered to be significant

Lower results obtained with the Tosoh G8 when samples contain the variant HbAS

Hemoglobin S (% variant: 31–42 % S)

See Addendum III on page 13 for NGSP conversion rate

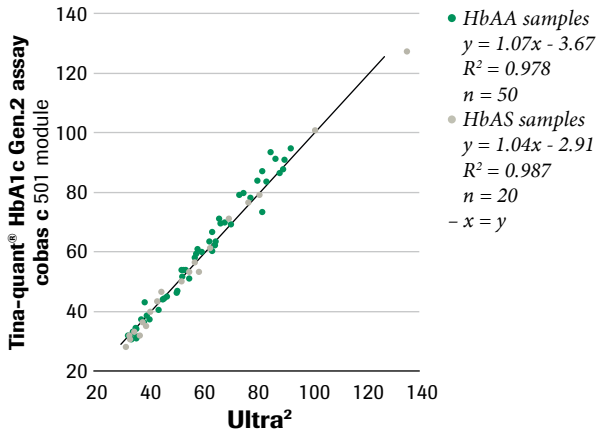


Fig. 3: Results from the Tina-quant® HbA1c Gen.2 assay on the **cobas c** 501 module in the presence of HbAS
HbA1c: 30–100 mmol/mol

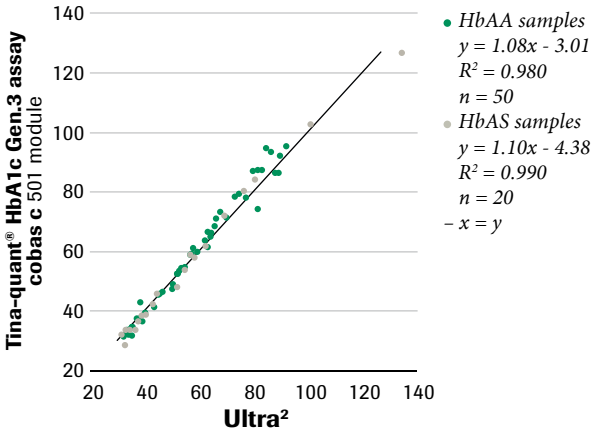


Fig. 4: Results from the Tina-quant® HbA1c Gen.3 assay on the **cobas c** 501 module in the presence of HbAS
HbA1c: 30–100 mmol/mol

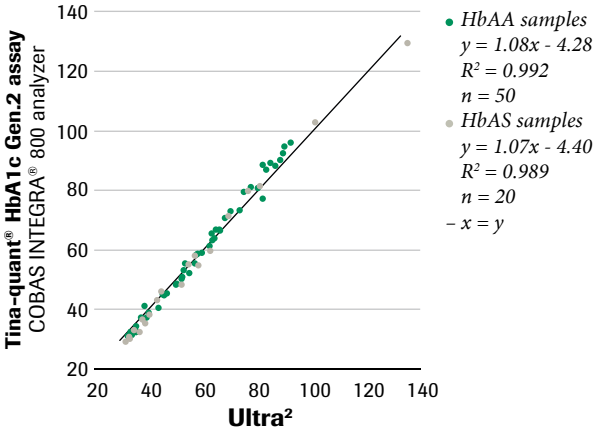


Fig. 5: Results from the Tina-quant® HbA1c Gen.2 assay on the COBAS INTEGRA® 800 analyzer in the presence of HbAS
HbA1c: 30–100 mmol/mol

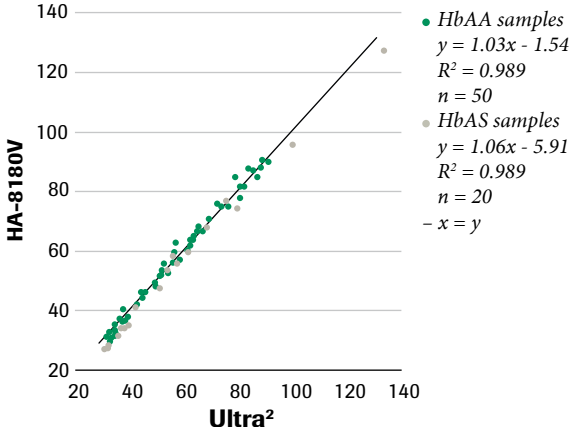


Fig. 6: Results from the Menarini HA-8180V in the presence of HbAS
HbA1c: 30–100 mmol/mol

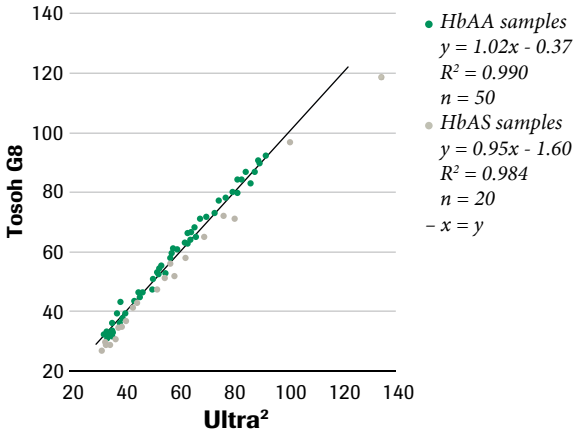


Fig. 7: Results from the Tosoh G8 in the presence of HbAS
HbA1c: 30–100 mmol/mol

All methods free from interference from HbAC

Hemoglobin C (% variant: 36–42 % C)

See Addendum III on page 13 for NGSP conversion rate

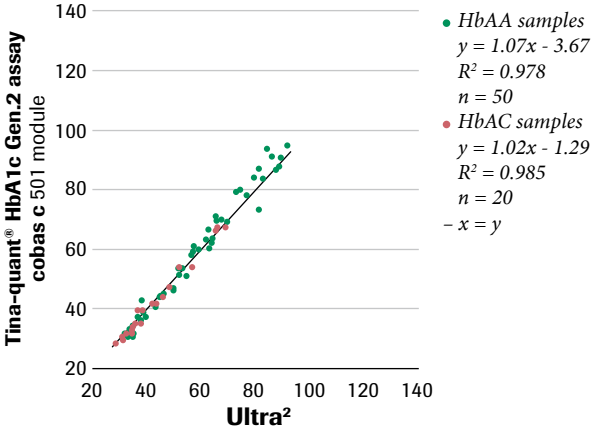


Fig. 8: Results from the Tina-quant® HbA1c Gen.2 assay on the **cobas c** 501 module in the presence of HbAC
HbA1c: 26–104 mmol/mol

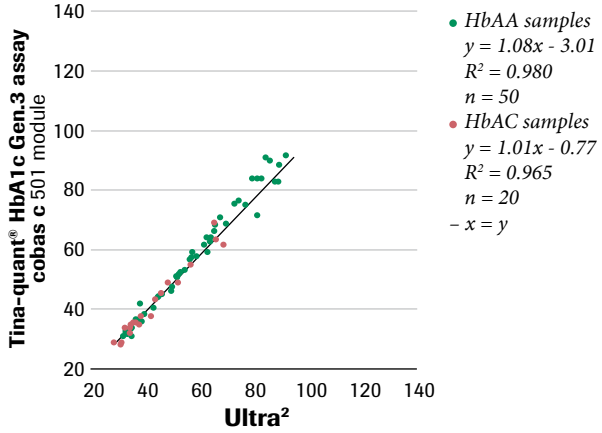


Fig. 9: Results from the Tina-quant® HbA1c Gen.3 assay on the **cobas c** 501 module in the presence of HbAC
HbA1c: 26–104 mmol/mol

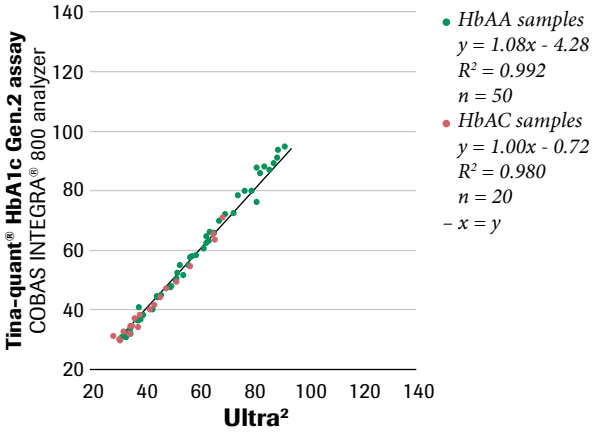


Fig. 10: Results from the Tina-quant® HbA1c assay Gen.2 on the COBAS INTEGRA® 800 analyzer in the presence of HbAC
HbA1c: 26–104 mmol/mol

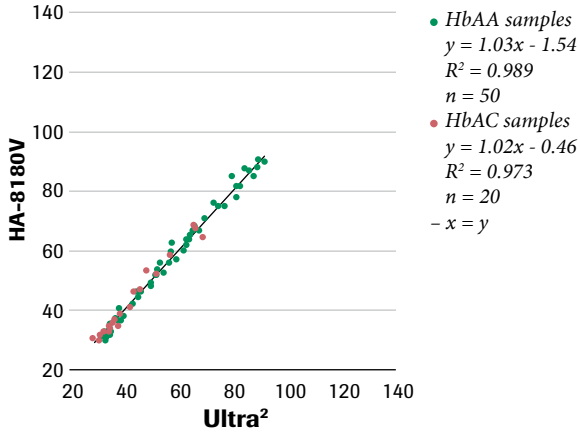


Fig. 11: Results from the Menarini HA-8180V in the presence of HbAC
HbA1c: 26–104 mmol/mol

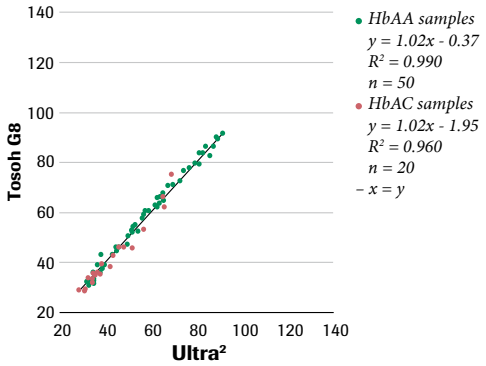


Fig. 12: Results from the Tosoh G8 in the presence of HbAC
HbA1c: 26–104 mmol/mol

Presence of HbAD causes no results on Menarini HA-8180V

Hemoglobin D (% variant: 37–42 % D)

See Addendum III on page 13 for NGSP conversion rate

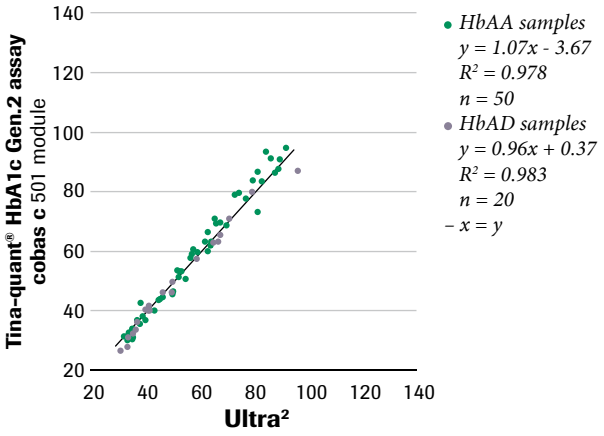


Fig. 13: Results from the Tina-quant® HbA1c Gen.2 assay on the **cobas c** 501 module in the presence of HbAD
HbA1c: 30–96 mmol/mol

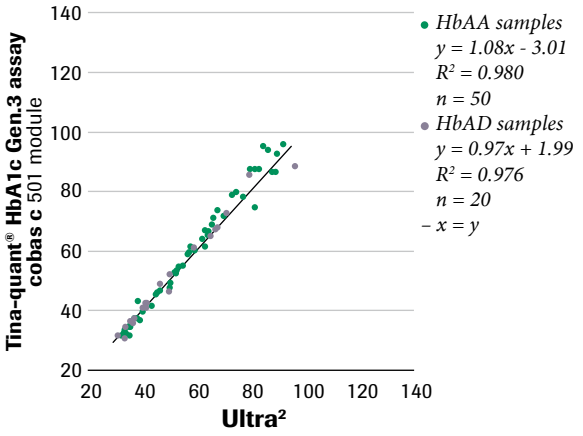


Fig. 14: Results from the Tina-quant® HbA1c Gen.3 assay on the **cobas c** 501 module in the presence of HbAD
HbA1c: 30–96 mmol/mol

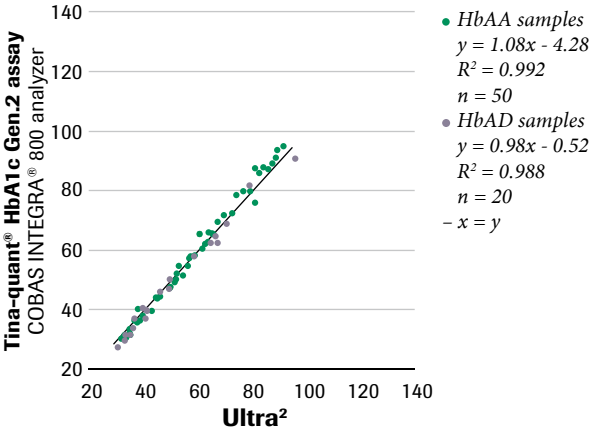
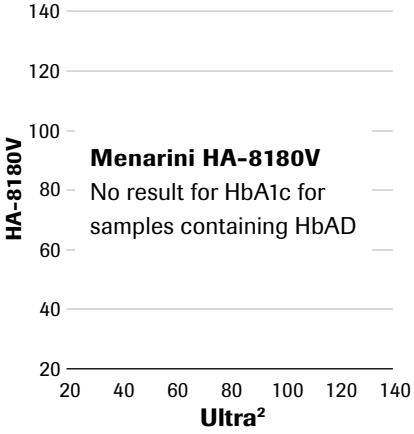


Fig. 15: Results from the Tina-quant® HbA1c Gen.2 assay on the COBAS INTEGRA® 800 analyzer in the presence of HbAD
HbA1c: 30–70 mmol/mol A2, 36-54 mmol/mol AJ, 34-43 mmol/mol AG



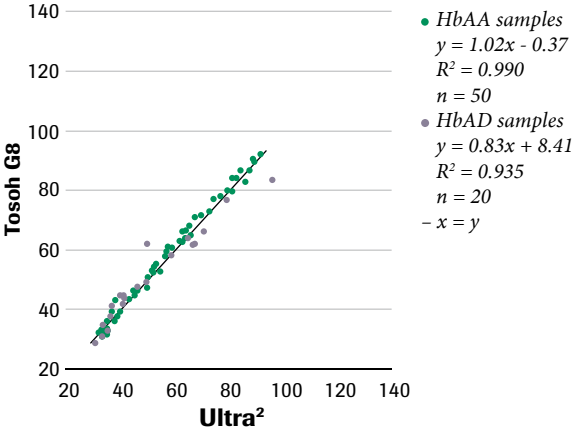


Fig. 16: Results from the Tosoh G8 in the presence of HbAD
HbA1c: 30–96 mmol/mol

No results on Menarini HA-8180V and false low values on Tosoh G8 in samples containing HbAE

Hemoglobin E (% variant: 27–33 % E)

See Addendum III on page 13 for NGSP conversion rate

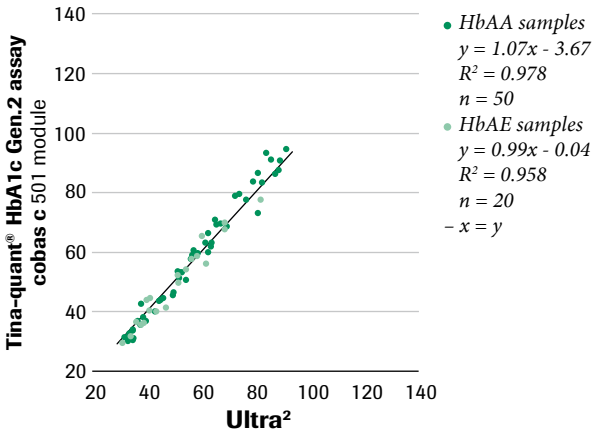


Fig. 17: Results from the Tina-quant® HbA1c Gen.2 assay on the **cobas c** 501 module in the presence of HbAE
HbA1c: 38–81 mmol/mol

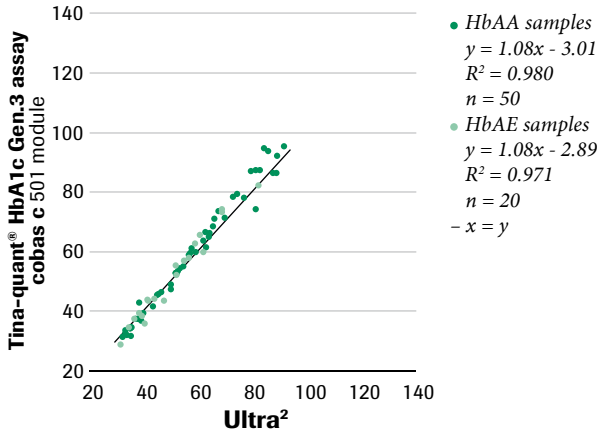


Fig. 18: Results from the Tina-quant® HbA1c Gen.3 assay on the **cobas c** 501 module in the presence of HbAE
HbA1c: 38–81 mmol/mol

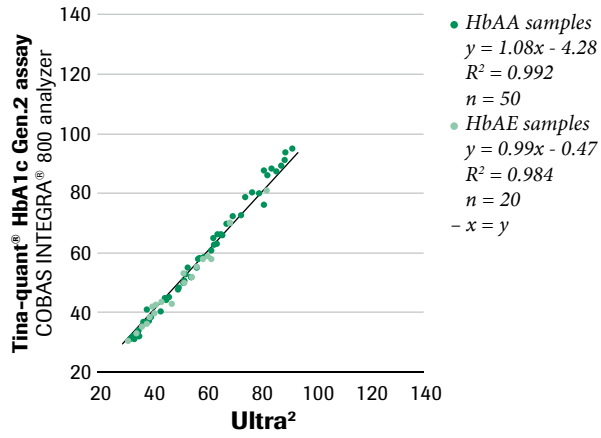
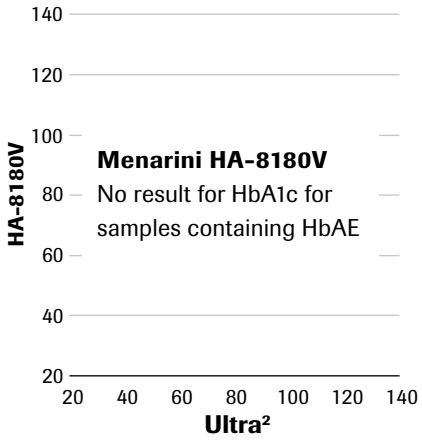


Fig. 19: Results from the Tina-quant® HbA1c Gen.2 assay on the COBAS INTEGRA® 800 analyzer in the presence of HbAE
HbA1c: 38–81 mmol/mol



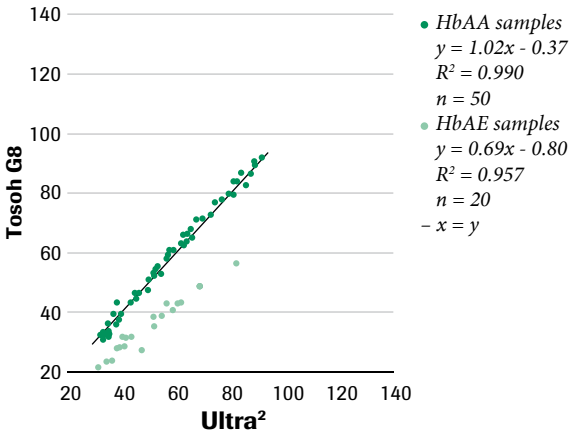


Fig. 20: Results from the Tosoh G8 in the presence of HbAE
HbA1c: 38–81 mmol/mol

Interference from HbAJ and HbAG is observed with Menarini HA-8180V and samples containing HbAJ gave false low results on Tosoh G8

Hemoglobin A2, AJ and AG (% variant: 4–7 % A2, 49–51 % AJ, approximately 18 % AG)

See Addendum III on page 13 for NGSP conversion rate

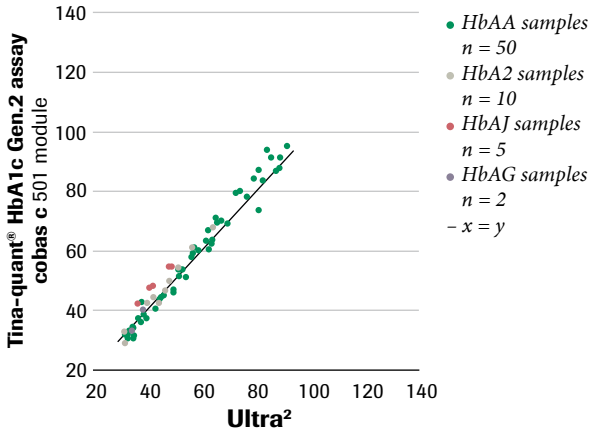


Fig. 21: Results from the Tina-quant® HbA1c Gen.2 assay on the **cobas c** 501 module in the presence of HbA2, HbAJ and HbAG
HbA1c: 30-70 mmol/mol A2, 36-54 mmol/mol AJ, 34-43 mmol/mol AG

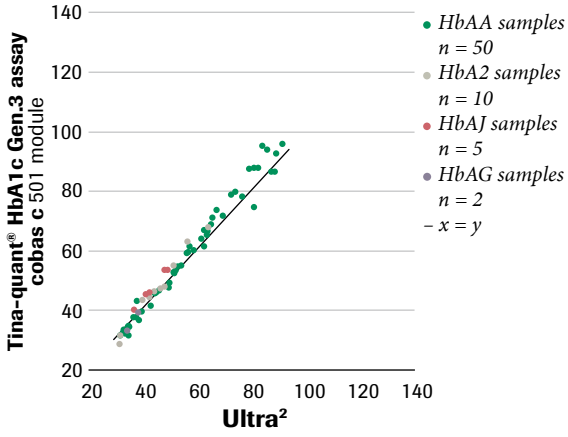


Fig. 22: Results from the Tina-quant® HbA1c Gen.3 assay on the **cobas c** 501 module in the presence of HbA2, HbAJ and HbAG
HbA1c: 30-70 mmol/mol A2, 36-54 mmol/mol AJ, 34-43 mmol/mol AG

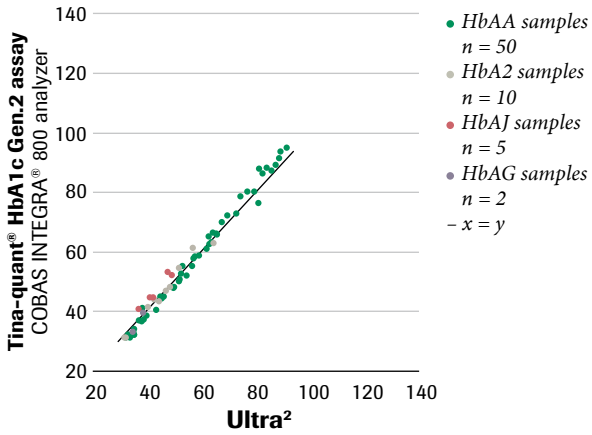


Fig. 23: Results from the Tina-quant® HbA1c Gen.2 assay on the COBAS INTEGRA® 800 analyzer in the presence of HbA2, HbAJ and HbAG
HbA1c: 30-70 mmol/mol A2, 36-54 mmol/mol AJ, 34-43 mmol/mol AG

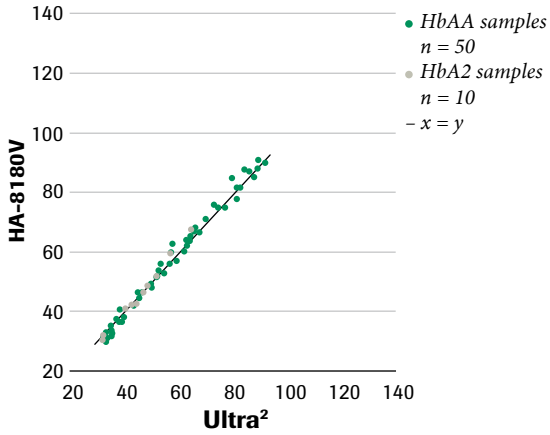


Fig. 24: Results from the Menarini HA-8180V in the presence of HbA2
HbA1c: 30-70 mmol/mol

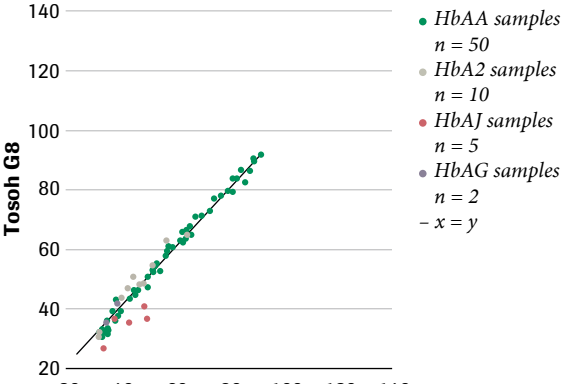


Fig. 25: Results from the Tosoh G8 in the presence of HbA2, HbAJ and HbAG
HbA1c: 30-70 mmol/mol A2, 36-54 mmol/mol AJ, 34-43 mmol/mol AG

All investigated rare variants except for Hb Indonesia and Hb Hopkins-2 gave an abnormal chromatogram and no HbA1c result with Menarini HA-8180V and Tosoh G8

Rare variants

See Addendum III on page 13 for NGSP conversion rate

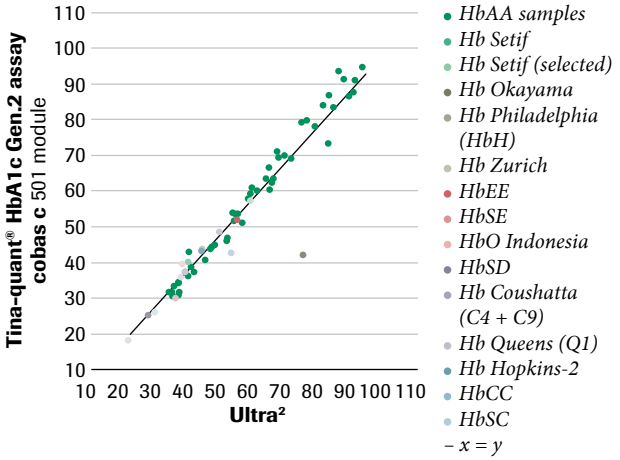


Fig. 26: Results from the Tina-quant® HbA1c Gen.2 assay on the **cobas c** 501 module in the presence of rare hemoglobin variants

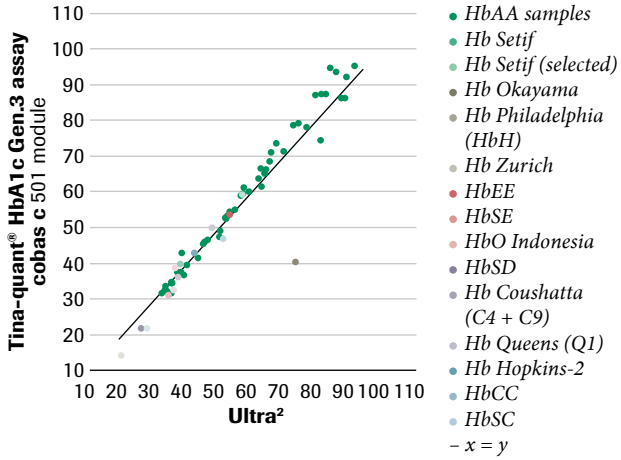


Fig. 27: Results from the Tina-quant® HbA1c Gen.3 assay on the **cobas c** 501 module in the presence of rare hemoglobin variants

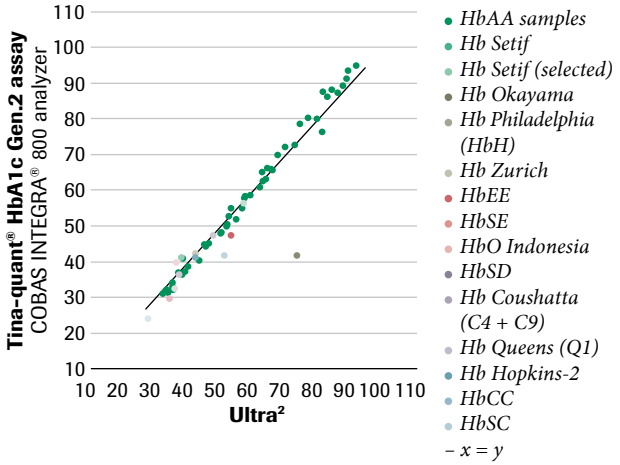
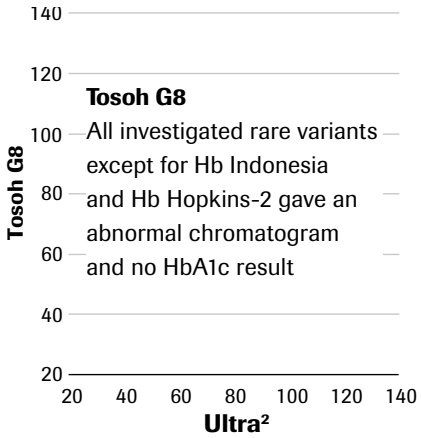
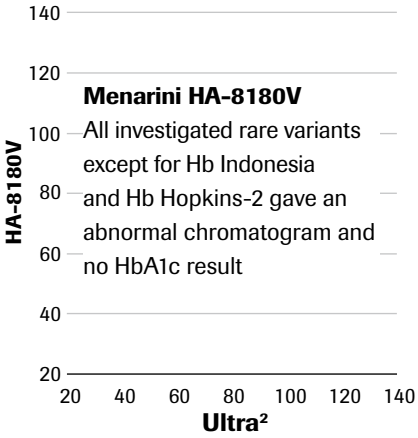


Fig. 28: Results from the Tina-quant® HbA1c Gen.2 assay on the COBAS INTEGRA® 800 analyzer in the presence of rare hemoglobin variants





Variant Type	HbA1c (mmol/mol)	n
HbAA	0-100	50
Hb Setif	38-58	2
Hb Okayama	74	1
Hb Philadelphia (HbH)	41	1
Hb Zurich	20	1
HbEE	60	1
HbSE	36	1
HbO Indonesia	39	1
HbSD	21	1
Hb Coushatta (C4 + C9)	*	2
Hb Queens	*	1
Hb Hopkins-2	43	1
HbCC	51	1
HbSC	21	1

** No indications possible*

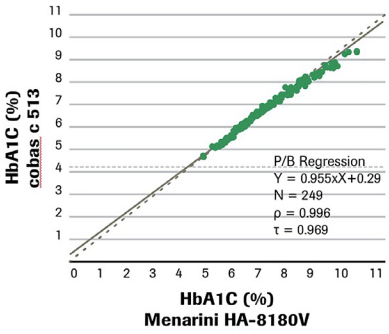
Table 3: Rare hemoglobin variants used on all systems

Addendum I

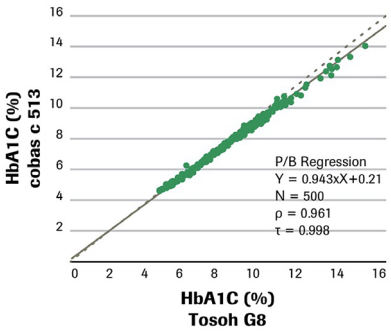
HbA1c Multi Center Evaluation Study – cobas c 513

The cobas c 513 analyzer shows excellent correlation to different HPLC systems in a method comparison utilizing whole blood samples

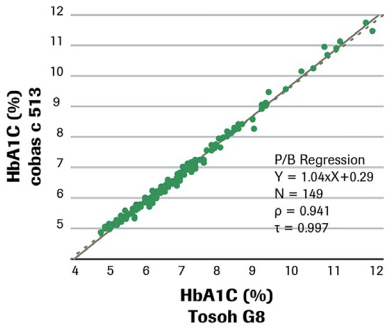
Lab 03, whole blood application cobas c 513 vs Menarini HA-8180V



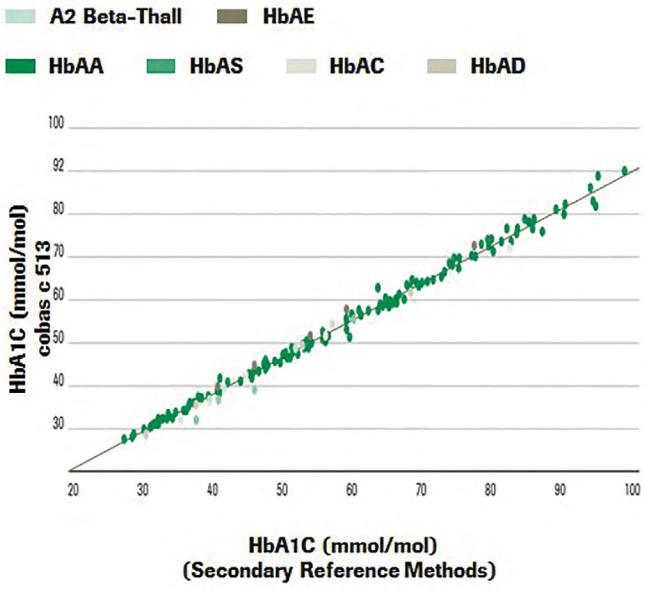
Lab 02, whole blood application cobas c 513 vs Tosoh G8 with IFCC SRM calib



cobas c 513 vs Tosoh G8 (NGSP values)



Tina-quant HbA1c delivers accurate results, displays no interference by common hemoglobin variants



Addendum II

Updated Statistics

As of February 2018, there are:

- 1,771 known hemoglobin mutations (hemoglobin variants and thalassemias).[7]
- 1,315 known human hemoglobin variants.[7]
- 19 variants with a mutation in the first four amino acids of the glycated hemoglobin chain.[7]

Addendum III

Conversion Factor – IFCC mmol/mol to NGSP %[8]

Conversion Factor Equation: NGSP = (0.09148*IFCC) + 2.152

IFCC mnol/mol	31	42	53	64	75	86	97	108
NGSP %	5.0	6.0	7.0	8.0	9.0	10.0	11.0	12.0

SOURCE: NGSP – Convert between NGSP, IFCC, and eAG units

Acknowledgement

This study was conducted by the European Reference Laboratory (IFCC/NGSP Reference Laboratory) Zwolle and Winterswijk, NL, supported by Roche.

1. NGSP. HbA1c methods: Effects of hemoglobin variants (HbC, HbS, HbE and HbD traits) and elevated fetal hemoglobin (HbF). Updated July 2013. Available at: http://www.ngsp.org/interf.asp (accessed October 2013).
2. HbVar. A database of human hemoglobin variants and thalassemias. Available at: http://globin.cse.psu.edu/globin/hbvar/ (accessed October 2013).
3. Consensus Committee. (2007). Consensus statement on the worldwide standardization of the hemoglobin A1C measurement: the American Diabetes Association, European Association for the Study of Diabetes, International Federation of Clinical Chemistry and Laboratory Medicine, and the International Diabetes Federation. *Diabetes Care* 30, 2399–2400.
4. Rohlfing, C.L., Connolly, S.M., England, J.D., Hanson, S.E., Moellering, C.M., Bachelder, J.R., Little, R.R. (2008). The effect of elevated fetal hemoglobin on hemoglobin A1c results: five common hemoglobin A1c methods compared with the IFCC reference method. *Am J Clin Pathol* 129, 811–814.
5. Little, R.R., Rohlfing, C.L., Hanson, S.E., Schmidt, R.L., Lin, C.N., Madsen, R.W., Roberts, W.L. (2012). The effect of increased fetal hemoglobin on 7 common Hb A1c assay methods. *Clin Chem* 58, 945–947.
6. HbVar. A database of human hemoglobin variants and thalassemias. Available at: http://globin.cse.psu.edu/globin/hbvar/ (accessed February 2018).
7. NGSP.Org. IFCC Standardization of HbA1c. 2010. Available at: http://www.ngsp.org/ADA.asp (accessed February 2018).

Roche Diagnostics
9115 Hague Road
Indianapolis, IN 46256
usdiagnostics.roche.com