Hemoglobin variant study
*Turbidimetry versus HPLC*

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**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 technicians 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.

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**Check every chromatogram**

Chromatogram normal*  \[---\]  
Chromatogram abnormal  \[---\]

Chromatogram same as chromatogram of a common Hb variant? (HbAS, HbAC, HbAD, HbAE)

Interference from common Hb variant with your HbA1c method? 
See for information: www.ngsp.org/interf.asp

No  \[---\]  
Yes  \[---\]

HbA1c can be reported  \[---\]  
Determine HbA1c with another method, preferably affinity chromatography

Is the variant heterozygous (HbA present)?

Yes  \[---\]  
No  \[---\]

Do not report HbA1c

* 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

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

When using an immunoassay, technicians 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.
For the vast majority of the more than 1,175 Hb variants identified to date, the mutation is not in the first four amino acids of the Hb protein and therefore does not interfere with the Tina-quanta 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).

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-quanta HbA1c Gen.2 and Gen.3 assays.

**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-quanta HbA1c Gen.2 on the COBAS INTEGRA® 800 analyzer; IFCC and NGSP certified immunoassay (Roche)
- Trinity Ultra®, IFCC and NGSP certified affinity chromatography HPLC (Trinity Biotech)
- Tosoh G8; IFCC certified cation-exchange HPLC (Tosoh Bioscience).
Site 2: IFFC/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. 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® 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, 8–11, 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 %. 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 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).

### Table 1: Results using Tosoh G8

<table>
<thead>
<tr>
<th></th>
<th>Linear regression</th>
<th>Deming regression</th>
<th>R²</th>
<th>42 mmol/mol</th>
<th>Significant difference</th>
<th>75 mmol/mol</th>
<th>Significant difference</th>
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<tbody>
<tr>
<td>AA</td>
<td>y = 1.08x - 3.01</td>
<td>y = 1.09x - 3.70</td>
<td>0.980</td>
<td>42</td>
<td>78</td>
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<td>AS</td>
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<td>76</td>
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<tr>
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<td>42</td>
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<tr>
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<td>y = 1.09x + 1.41</td>
<td>0.976</td>
<td>43</td>
<td>No</td>
<td>75</td>
<td>No</td>
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<tr>
<td>AE</td>
<td>y = 1.08x - 2.89</td>
<td>y = 1.10x - 3.76</td>
<td>0.971</td>
<td>42</td>
<td>No</td>
<td>78</td>
<td>No</td>
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<tr>
<td>HbF</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>&gt; HbF 8 %</td>
<td></td>
<td></td>
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<tr>
<td>A2</td>
<td></td>
<td></td>
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<tr>
<td>Rare variants</td>
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### Table 2: Results using Tosoh G8

<table>
<thead>
<tr>
<th></th>
<th>Linear regression</th>
<th>Deming regression</th>
<th>R²</th>
<th>42 mmol/mol</th>
<th>Significant difference</th>
<th>75 mmol/mol</th>
<th>Significant difference</th>
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</thead>
<tbody>
<tr>
<td>AA</td>
<td>y = 1.08x - 4.28</td>
<td>y = 1.08x - 4.58</td>
<td>0.992</td>
<td>41</td>
<td>77</td>
<td>No</td>
<td>No</td>
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<tr>
<td>AS</td>
<td>y = 1.07x - 4.40</td>
<td>y = 1.08x - 4.72</td>
<td>0.989</td>
<td>41</td>
<td>No</td>
<td>76</td>
<td>No</td>
</tr>
<tr>
<td>AC</td>
<td>y = 1.00x - 0.72</td>
<td>y = 1.01x - 1.07</td>
<td>0.980</td>
<td>41</td>
<td>No</td>
<td>75</td>
<td>No</td>
</tr>
<tr>
<td>AD</td>
<td>y = 0.98x - 0.52</td>
<td>y = 1.00x - 0.89</td>
<td>0.988</td>
<td>41</td>
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<td>73</td>
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<tr>
<td>AE</td>
<td>y = 0.99x - 0.47</td>
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<td>&gt; HbF 8 %</td>
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<td>HbF</td>
<td></td>
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<td>A2</td>
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<td>Rare variants</td>
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### Table 3: Results using Tosoh G8

<table>
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<tr>
<th></th>
<th>Linear regression</th>
<th>Deming regression</th>
<th>R²</th>
<th>42 mmol/mol</th>
<th>Significant difference</th>
<th>75 mmol/mol</th>
<th>Significant difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>y = 1.08x - 3.67</td>
<td>y = 1.09x - 4.42</td>
<td>0.978</td>
<td>41</td>
<td>77</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>AS</td>
<td>y = 1.04x - 2.91</td>
<td>y = 1.05x - 3.26</td>
<td>0.987</td>
<td>41</td>
<td>No</td>
<td>75</td>
<td>No</td>
</tr>
<tr>
<td>AC</td>
<td>y = 1.02x - 1.29</td>
<td>y = 1.03x - 1.64</td>
<td>0.985</td>
<td>42</td>
<td>No</td>
<td>76</td>
<td>No</td>
</tr>
<tr>
<td>AD</td>
<td>y = 0.96x + 0.37</td>
<td>y = 0.97x - 0.05</td>
<td>0.983</td>
<td>41</td>
<td>No</td>
<td>72</td>
<td>No</td>
</tr>
<tr>
<td>AE</td>
<td>y = 0.99x - 0.04</td>
<td>y = 1.00x - 0.44</td>
<td>0.958</td>
<td>42</td>
<td>No</td>
<td>74</td>
<td>No</td>
</tr>
<tr>
<td>HbF</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>&gt; HbF 8 %</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A2</td>
<td></td>
<td></td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Rare variants</td>
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</tbody>
</table>
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 plus (hopefully) the associated checking algorithm to avoid incorrect results. Hence, the advantages/disadvantages of the different HbA1c methods will be viewed differently by different laboratories.

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

<table>
<thead>
<tr>
<th>Menarini HA-8180V</th>
<th>Tosoh G8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linear regression</td>
<td>Linear regression</td>
</tr>
<tr>
<td>AA $y = 1.03x - 1.54$</td>
<td>$y = 1.03x - 1.90$</td>
</tr>
<tr>
<td>AS $y = 1.06x - 5.91$</td>
<td>$y = 1.07x - 6.31$</td>
</tr>
<tr>
<td>AC $y = 1.02x - 0.46$</td>
<td>$y = 1.03x - 1.08$</td>
</tr>
<tr>
<td>AD</td>
<td></td>
</tr>
<tr>
<td>AE</td>
<td></td>
</tr>
<tr>
<td>HbF</td>
<td></td>
</tr>
<tr>
<td>A2</td>
<td></td>
</tr>
<tr>
<td>Rare variants</td>
<td></td>
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</tbody>
</table>
Figure 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

Figure 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

Figure 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

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

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

Lower results obtained with the Tosoh G8 when samples contain the variant HbAS
Hemoglobin S (% variant: 31–42 % S)
All methods free from interference from HbAC

Hemoglobin C (% variant: 36–42 % C)

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

- HbAA samples
  y = 1.07x - 3.67
  R² = 0.978
  n = 50

- HbAC samples
  y = 1.02x - 1.29
  R² = 0.985
  n = 20

- x = y

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

- HbAA samples
  y = 1.08x - 3.01
  R² = 0.980
  n = 50

- HbAC samples
  y = 1.01x - 0.77
  R² = 0.965
  n = 20

- x = y

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

- HbAA samples
  y = 1.08x - 4.28
  R² = 0.992
  n = 50

- HbAC samples
  y = 1.00x - 0.72
  R² = 0.980
  n = 20

- x = y

Fig. 11: Results from the Menarini HA-8180V in the presence of HbAC

HbA1c: 26–104 mmol/mol

- HbAA samples
  y = 1.03x - 1.54
  R² = 0.989
  n = 50

- HbAC samples
  y = 1.02x - 0.46
  R² = 0.973
  n = 20

- x = y

Fig. 12: Results from the Tosoh G8 in the presence of HbAC

HbA1c: 26–104 mmol/mol

- HbAA samples
  y = 1.02x - 0.37
  R² = 0.990
  n = 50

- HbAC samples
  y = 1.02x - 1.95
  R² = 0.960
  n = 20

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

Hemoglobin D (% variant: 37–42 % D)

Fig. 13: Results from the Tina-quant® HbA1c Gen.2 assay on the COBAS INTEGRA® 800 analyzer in the presence of HbAD
HbA1c: 30–96 mmol/mol

HbAA samples
\[ y = 1.08x - 3.67 \]
\[ R^2 = 0.978 \]
\[ n = 50 \]

HbAD samples
\[ y = 0.96x + 0.37 \]
\[ R^2 = 0.983 \]
\[ n = 20 \]

\[ -x = y \]

Fig. 14: Results from the Tina-quant® HbA1c Gen.3 assay on the COBAS INTEGRA® 800 analyzer in the presence of HbAD
HbA1c: 30–96 mmol/mol

HbAA samples
\[ y = 1.08x - 3.01 \]
\[ R^2 = 0.980 \]
\[ n = 50 \]

HbAD samples
\[ y = 0.97x + 1.99 \]
\[ R^2 = 0.976 \]
\[ n = 20 \]

\[ -x = y \]

Fig. 15: Results from the Tina-quant® HbA1c Gen.2 assay on the COBAS INTEGRA® 800 analyzer in the presence of HbAD
HbA1c: 30–96 mmol/mol

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

HbAA samples
\[ y = 1.02x - 0.37 \]
\[ R^2 = 0.990 \]
\[ n = 50 \]

HbAD samples
\[ y = 0.83x + 8.41 \]
\[ R^2 = 0.935 \]
\[ n = 20 \]

\[ -x = y \]
No results on Menarini HA-8180V and false low values on Tosoh G8 in samples containing HbAE

Hemoglobin E (% variant: 27–33 % E)

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)**

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**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**

- HbAA samples: 50
- HbA2 samples: 10
- HbAJ samples: 5
- HbAG samples: 2

---

**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**

- HbAA samples: 50
- HbA2 samples: 10
- HbAJ samples: 5
- HbAG samples: 2

---

**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**

- HbAA samples: 50
- HbA2 samples: 10
- HbAJ samples: 5
- HbAG samples: 2

---

**Fig. 24: Results from the Menarini HA-8180V in the presence of HbA2**

- HbAA samples: 50
- HbA2 samples: 10
- HbAJ samples: 5
- HbAG samples: 2

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**Fig. 25: Results from the Tosoh G8 in the presence of HbA2, HbAJ and HbAG**

- HbAA samples: 50
- HbA2 samples: 10
- HbAJ samples: 5
- HbAG samples: 2
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

Table 3: Rare hemoglobin variants used on all systems
Acknowledgement
This study was conducted by the European Reference Laboratory (IFCC/NGSP Reference Laboratory) Zwolle and Winterswijk, NL, supported by Roche.

References