Hemoglobin A1c (Hb A1c): Hb A1c is measured at the Clinical Chemistry Laboratory at Fletcher Allen Health Care, an affiliate of the University of Vermont. HbA1c is the most quantitatively present complex among the glycohemoglobins. HBA1c is nonenzymatically synthesized in two steps forming a stable ketoamine of the hemoglobin complex (SA1c). The Tosoh G7 Automated HPLC – HbA1c Variant Analysis Mode uses non-porous ion exchange, high performance liquid chromatography (HPLC) and microcomputer technology to measure the HbA1c as a percentage of the total amount of hemoglobin present in the sample. Separation is achieved on a TSKgel G7 HSi Variant Column using ionic interactions by the cation exchange group on the column resin and the hemoglobin components. The hemoglobin fractions (designated as A1a, A1b, F, $LA1c^{+}$, SA1c, A0 and H-V0, H-V1, H-V2) are subsequently removed from the column by stepwise elution. Retention time is calculated by the G7 Automated HPLC software. The separated hemoglobin components pass through the LED photometer flow cell where the analyzer measures change in absorbance at 415nm. The analyzer calculates the relative percentages of each hemoglobin fraction. The Tosoh G7 Automated HPLC – HbA1c Variant Analysis Mode is certified by the National Glycohemoglobin Standardization Program (NGSP) and the final report it traceable to the Diabetes Control and Complications Trial (DCCT). The intra- and inter-assay CVs range from 0.5-0.9% and 0.7-1.6%, respectively. <6% HbA1c is considered to be in the normal range. The A1c goal for nonpregnant adults in general is <7%. The A1c goal for selected individual patients is as close to normal (<6%) as possible without significant hypoglycemia.

Abnormal Hemoglobins on the Tosoh G7:

There are quite a few hemoglobinopathies that we can get a preliminary identification of on the Tosoh G7. All of these however, need to be confirmed by Hgb Electrophoresis, as the Tosoh G7 uses HPLC and may of these hgbs can elute at very similar retention times. The hemoglobinopathies include Hgb S, C, D, E, G, and fast migrating hgbs such as J. We will also be able to tell if they are present in the heterozygous (trait) or homozygous (disease) state. FAHC abnormal results are reported on the following variants: C, D, E, G, J.

None of the hgbs in the heterozygous state are clinically or hematologically harmful; except that Hgb E usually mimics a thalassemic picture causing an increased RBC and decreased MCV. One reason a patient might be interested in knowing about any of these hemoglobinopathies would be for genetic, family planning reasons for themselves or their children (2 heterozygotes could pass on their hemoglobinopathies to get a homozygote or a double heterozygote – which would be a problem).

All of the hemoglobinopathies mentioned except S and C trait impact the accuracy of the Hgb A1C result. The impact will be a falsely decreased Hgb A1C result. When the Fletcher Allen Chemistry Laboratory has any abnormal hgb present other than S or C, they do not report the A1c result.

Elevated Hgb F values:
We do not generally worry about HgbF values until they are between 4-5% or greater. The clinical importance of an elevated Hgb F varies and needs to be looked at with the rest of that patient's clinical picture. Some causes of elevated Hgb F levels can be any kind of stress on the bone marrow such as certain medications, myelodysplastic syndromes, hemolytic anemias or and disease impairing erythropoiesis. Late pregnancy can also cause slightly elevated Hgb F levels. Hereditary persistence of Fetal Hemoglobin (HPFH) and Deta-Beta thalassemia will cause higher HgbF levels (greater than 10%).

Application Instruction Guide

G7 Automated HPLC – HbA1c Variant Analysis Mode

Intended Use

The Tosoh G7 Automated HPLC – HbA1c Variant Analysis Mode is intended for *in vitro* diagnostic use for the measurement of hemoglobin A1c (HbA1c) in whole blood specimens. Hemoglobin A1c measurements are used in the clinical management of diabetes to assess the long-term efficacy of diabetic control.

The procedure is specifically designed for the Tosoh G7 Automated HPLC Analyzer equipped with appropriate software, TSKgel G7 HSi Column, Elution Buffers, and Hemolysis & Wash Solution.

The analyzer uses non-porous ion exchange, high performance liquid chromatography (HPLC) and microcomputer technology to quickly and accurately measure the HbA1c as a percentage of the total amount of hemoglobin present in the sample.

Summary and Explanation of the Test

Diabetes causes elevated levels of glucose to circulate in the blood. Maintaining normal levels of blood glucose is part of the routine clinical management of diabetes. Continuous and careful management of blood glucose levels prevents development of serious long term complications resulting from vascular impairment such as retinopathy, nephropathy, and neuropathy.

Although a fasting blood glucose measurement gives the clinician information about the patient's status over the last twelve hours, the stable HbA1c offers a more accurate indication of the patient's long-term diabetic control over the last two to three months.

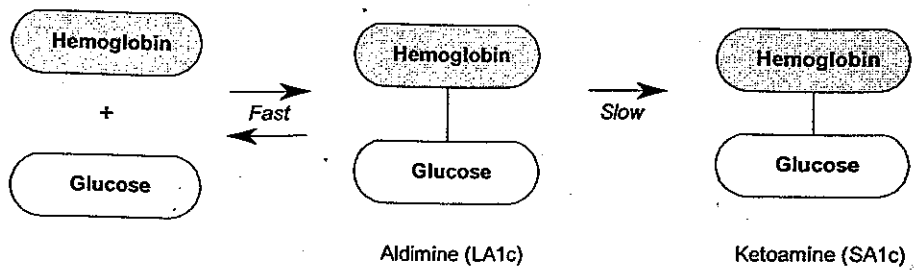
Glycohemoglobin is a general term for hemoglobin-glucose complexes in which glucose is bound to the alpha and beta chains of hemoglobin. The most quantitatively prevalent complex is called HbA1c, in which glucose binds to the N-terminus of the beta chain of Hb A.

HbA1c is nonenzymatically synthesized in two steps:

1. The glucose aldehyde group and the free amino group on the valine in the N-terminus of the hemoglobin beta chain react to form the Schiff base, aldimine (also known as labile HbA1c or LA1c).

2. A stable ketoamine form of the hemoglobin complex (SA1c) is then produced by a reaction known as Amadori rearrangement.

The level of LA1c changes rapidly in response to changes in blood glucose concentration. However, the level of the SA1c does not fluctuate significantly in response to physiological factors. Consequently, the SA1c measurement provides a better indication of the average glucose level over the previous two to three months (the average red blood cell life span).



Formation of Labile and Stable Forms of A1c (LA1c and SA1c)

In the past, accurate measurement of SA1c was possible only after removing LA1c by pretreatment. As with the Tosoh A1c 2.2 Plus Glycohemoglobin Analyzer, the Tosoh G7 Automated HPLC Analyzer – HbA1c Variant Analysis Mode can individually resolve SA1c and LA1c on the chromatogram without manual pretreatment, allowing accurate measurement of SA1c directly.

Principle of the Assay

The G7 Automated HPLC Analyzer – HbA1c Variant Analysis Mode uses non-porous ion-exchange high performance liquid chromatography (HPLC) for rapid, accurate, and precise separation of the stable form of HbA1c from other hemoglobin fractions. Analysis is carried out without off-line specimen pretreatment or interference from Schiff base.

The analyzer dilutes the whole blood specimen with Hemolysis & Wash Solution, and then injects a small volume of this specimen onto the TSKgel G7 HSi Variant Column. Separation is achieved by utilizing differences in ionic interactions between the cation exchange group on the column resin surface and the hemoglobin components. The hemoglobin fractions (designated as A1a, A1b, F, LA1c+, SA1c, A0, and H-V0, H-V1, H-V2) are subsequently removed from the column by performing a step-wise elution using the varied salt concentrations in the Elution Buffers HSi Variant 1, 2, and 3.

The time from injection of the sample to the time the specific peak elutes off the column is called Retention Time. The G7 Automated HPLC software has been written so that each of the expected fractions has a window of acceptable retention times. If the designated peak falls within the expected window, the chromatogram peaks will be properly identified. When a peak elutes at a retention time not within a specified window, an unknown peak (P00) results. If more than one peak elutes at times not specified by the software windows, each is given a sequential P0x title. In order to keep the peaks within their appropriate windows, it may be necessary to change how fast the buffers are moving through the system by changing the pump flow rate.

The separated hemoglobin components pass through the LED photometer flow cell where the analyzer measures changes in absorbance at 415 nm. The analyzer integrates and reduces the raw data, and then calculates the relative percentages of each hemoglobin fraction. The Total Area of the SA1c is divided by the sum of the total areas of all peaks up to and including the A0 to obtain a raw SA1c percentage. This uncorrected result is substituted as the "x" value in the linear regression formula determined during calibration. The analyzer prints the final numerical results and plots a chromatogram showing changes in absorbance versus retention time for each peak fraction.

The Tosoh G7 Automated HPLC Analyzer – HbA1c Variant Analysis Mode is certified by the National Glycohemoglobin Standardization Program (NGSP). The final reportable result is traceable to the Diabetes Control and Complications Trial (DCCT).

Performance Characteristics

Accuracy

Dilution / Linearity

Packed red blood cells from a normal specimen collected in EDTA were diluted with Hemolysis & Wash Solution and assayed. The study demonstrates that the assay is linear in samples with Total Area values from 500 to 3000.

Dilution Ratio	Total Area	SA1c %				%CV
		1	2	3	Mean	
600	468.0	4.1	4.1	4.2	4.1	1.1
400	687.0	4.1	4.2	4.1	4.1	1.1
300	829.0	4.1	4.1	4.1	4.1	0.0
250	927.0	4.1	4.1	4.1	4.1	0.0
200	1158.0	4.1	4.1	4.1	4.1	0.0
175	1327.0	4.1	4.1	4.0	4.1	1.2
150	1593.0	4.1	4.1	4.1	4.1	0.0
125	1815.0	4.1	4.1	4.1	4.1	0.0
113	2016.0	4.1	4.1	4.1	4.1	0.0
100	2266.0	4.1	4.1	4.1	4.1	0.0
50	3921.0	4.1	4.1	4.1	4.1	0.0

Recovery / Linearity

Two studies were conducted to demonstrate linearity in the measurement of % HbA1c. In the first study, a high and a low sample, both collected in EDTA, were mixed in specified ratios to produce 5 samples with a range of % HbA1c levels. In the first study, the two samples measured initially, 4.2 % and 20.8 % HbA1c.

HbA1c High Sample	HbA1c Low Sample	Theoretical Value (%)	Measured Value (%)	Percent Recovery
0.0 mL	1.0 mL	4.2	4.2	100
0.3 mL	0.7 mL	9.2	9.3	101
0.5 mL	0.5 mL	12.5	12.7	102
0.7 mL	0.3 mL	15.8	16.1	102
1.0 mL	0.0 mL	20.8	20.8	100

In the second study, a commercially available linearity product was used to demonstrate linearity.

Theoretical Value (%)	Measured Value (%)	Percent Recovery
3.0	3.0	100
5.4	5.4	100
10.0	10.3	103
18.4	18.9	103

Comparative Analysis

Patient specimens collected in EDTA were analyzed with the G7 Automated HPLC HbA1c Variant Analysis Mode (Y) and another commercially available HPLC glycohemoglobin assay (X). Least-squares linear regression analysis yielded the following correlation statistics between these two methods.

Slope:	1.001
Y-Intercept:	-0.12
Correlation Coefficient:	0.9987
Range of Samples (%):	3.4–15.9
n:	230

Precision

Intra-Assay

The intra-assay (within run) precision coefficient of variation was evaluated in five samples with three HbA1c levels (low HbA1c <6%, medium HbA1c 6–10%, and high HbA1c >10%). Three instrument sample modes were tested: diluted commercial control material, diluted whole blood and whole blood. The whole blood samples were collected in EDTA.

	Number of Replicates	Mean (%HbA1c)	Standard Deviation	Coefficient of Variation (%)
Control (L)	20	4.9	0.04	0.8
Control (H)	20	8.7	0.04	0.5
Diluted Blood	20	4.0	0.03	0.9
Whole Blood	20	4.1	0.02	0.5
Whole Blood	20	13.35	0.12	0.9

Inter-Assay

The inter-assay (between run) precision coefficient of variation was evaluated by testing two control specimens (HbA1c <6% and HbA1c >8%) in 20 runs.

	Number of Replicates	Mean (%HbA1c)	Standard Deviation	Coefficient of Variation (%)
Control A	20	6.4	0.10	1.6
Control B	20	11.01	0.08	0.7

Total Precision / Reproducibility

As part of a study done for NGSP Certification, NCCLS EP5-A protocol was run for 20 days with 2 runs per day with 2 replicates each to analyze patient pools (collected in EDTA) representing both non-diabetic and diabetic levels of HbA1c. The resulting coefficients of variation (%CV) are shown in the following table:

	Mean Value = 5.8%	Mean Value = 10.9%
Within Run Precision (CV)	0.90%	0.53%
Between Day Precision (CV)	0.40%	0.46%
Between Run Precision (CV)	0.52%	0.12%
Total Precision (CV)	1.12%	0.71%

Interference

Interference is defined, for the purposes of this study, to be recovery outside of 10% of the known specimen mean concentration.

- Icterus, as indicated by free and conjugated bilirubin concentrations up to 18.0 and 20.0 mg/dL, respectively, does not interfere with the assay.
- Lipemia, as indicated by triglyceride concentrations up to 2000 mg/dL, does not interfere with the assay.
- Concentrations of up to 20 mg/dL of sodium cyanate and acetaldehyde do not interfere with the assay.