

# Comparison of HbA1c and Glycated Protein Methodologies

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## LEARNING OBJECTIVES

1. Describe the role of HbA1c in the diagnosis and management of diabetes mellitus.
2. Describe the principle of immunoassay, affinity chromatography, high-pressure liquid chromatography, boronate affinity chromatography and isoelectric focusing for HbA1c measurements and the effects of hemoglobin variants on each methodology.
3. Identify genetic variants and their effect on HbA1c measurements.
4. Describe the drawbacks on point-of-care testing for HbA1c.
5. Discuss the use of other glycated protein testing.

**ABBREVIATIONS:** HbA1c - Hemoglobin A1c, eAG - Estimated average glucose, EDTA - Ethylenediaminetetraacetic Acid, HPFH - Hereditary persistent fetal hemoglobin, HPLC - High-pressure liquid chromatography, IEF - Isoelectric focusing, pI - Isoelectric point, NGSP - National Glycohemoglobin Standardization Program, DCCT - Diabetes Control and Complications Trial, POCT - Point-of-care testing, SNPs - Single nucleotide polymorphisms, CLSI - Clinical Laboratory Standard Institute, GA - Glycated Albumin, HbF - Fetal hemoglobin, RBC - Red blood cell, IFCC - International Federation of Clinical Chemistry

**INDEX TERMS:** HbA1c Testing, Hemoglobin Variants, HbA1c Methodologies, Standardization for HbA1c, Hb variants and HbA1c Measurements, Point-of-care Testing, Glycated Albumin Testing

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## INTRODUCTION

Managing diabetes mellitus requires consistent regulation and monitoring of blood glucose levels. HbA1c serves as a measurement for the diagnosis and monitoring of diabetes mellitus patients by determining long-term blood glucose control. Various methodologies of measuring HbA1c are utilized in laboratories throughout the world and may not produce equivalent results based on the effects of hemoglobin variants present in patients. The need to standardize HbA1c methodologies based on the effects of these variants is apparent. Point-of-care testing, as well as the measurement of different glycated proteins, can be examined as possible alternatives to the conventional HbA1c measurements.

The management of diabetes mellitus focuses on controlling the blood glucose levels. Consistent maintenance of the blood glucose concentration to levels at or near the nondiabetic range is optimal. It is also important to avoid significant fluctuations in the blood glucose. HbA1c serves as a longitudinal measurement for comparison of diabetes mellitus control over time. Glycosylated hemoglobin is measured to provide a picture of the overall management of blood glucose over the past 2 to 3 months.<sup>1</sup> Elevated HbA1c levels have been correlated with an increased risk for type 2 diabetes mellitus, as well as cardiovascular disease.<sup>2</sup> Measurements are taken at 2-3 month intervals, as that is the normal lifespan of red blood cells (RBC). HbA1c levels also are determined by the rate of erythrocyte synthesis/death. The life of RBC of diabetic patients is decreased, therefore presenting the possibility of falsely decreased

HbA1c measurements, which cannot be accounted for in any HbA1c analyzer by simply changing methodology.<sup>3</sup> Renal failure, hemoglobin variants, hematologic disorders and pregnancy all are characterized by increased RBC turnover, and therefore, inaccurate HbA1c measurements.<sup>4</sup> Commonly, HbA1c values are reported as a percentage. HbA1c is produced when normal adult hemoglobin (hemoglobin A) becomes bound to a glucose molecule to either one, or both, valines at the N-terminals of its  $\beta$ -polypeptide chains. The average glucose concentration and the life span of RBC in circulation are the two determinants of HbA1c. For every 1% change in HbA1c, the mean plasma glucose concentration increases by about 30 mg/dL (-1.7 mmol/L).<sup>1</sup> Normal HbA1c readings are below 5.7% (~39 mmol/mol) HbA1c readings between 5.7% and 6.4% (~39-46 mmol/mol) indicate prediabetes and the need for intervention to prevent the development of full diabetes mellitus. HbA1c readings of 6.5% (~48 mmol/mol) or above indicates diabetes and, according to the American Diabetes Association and an International Expert Committee, can be used alone to diagnose diabetes mellitus.<sup>5</sup> HbA1c is measured twice a year in diabetic patients with consistently healthy blood-glucose levels. In patients without consistent glycemic control, or those whose therapy is being altered, HbA1c is measured four times a year.<sup>1</sup> Clinical research suggests that two

individuals could have identical “glucose profiles” over an extended period of time, yet have different HbA1c levels. This variation in readings is based on various factors including lipid peroxidation, deglycation of glycated proteins and variations in genes that affect RBC life span. The HbA1c score can be converted to an estimated average glucose (eAG) value for comparison with glucose measurements. Conversion of HbA1c measurements to eAG is not conventionally practiced, although some argue its validity in helping patients relate their HbA1c values to glucose readings, which patients are more familiar with.<sup>4</sup>

**HbA1c testing methodologies**

HbA1c testing requires whole blood from samples collected with ethylenediaminetetraacetic acid (EDTA) as an anticoagulant. Measurement methods are either based on differences in charge or differences in structure between glycosylated and non-glycosylated hemoglobin molecules. Cation-exchange chromatography, electrophoresis and isoelectric focusing are HbA1c methodologies based on charge differences. Affinity chromatography and immunoassay methodologies harness the use of structural differences found in glycosylated HbA1c.<sup>1</sup> A summary of the methodologies is presented in Table 1.

**Table 1.** Comparison of Various HbA1c Measurement Methodologies.<sup>1,6</sup>

Method	Basis	Advantages	Disadvantages
Immunoassay	Structural differences. Uses antibodies toward glycated N-terminal of hemoglobin’s $\beta$ chain	Provide quantitative measurements of HbA1c	Hb F produces falsely low results. Hemoglobin variants such as Hb S and Hb C may cause false increase in HbA1c measurements
Affinity Chromatography	Structural differences. Uses borate to bind glycosylated hemoglobin based on HbA1c’s chemical structure	Not temperature-dependent. Usually not affected by variant hemoglobin F, S or C	High levels of HbF seen in thalassemia, HPFH, etc. may interfere with results
Ion-exchange Chromatography (Including HPLC)	Charge differences. A positively charged resin bed separates negatively charged HbA1c	HPLC has the advantage of separating hemoglobin variants into peaks	Highly temperature-dependent. Some hemoglobin variants may interfere with results.
Electrophoresis	Charge differences.	Separates various hemoglobin variants and allows for identification of variants	Hemoglobin F over 7% interferes with results
Isoelectric Focusing	Charge differences. Variation of electrophoresis using an isoelectric point to separate hemoglobin variants.	Sensitive, Economical, Quick	Pre-HbA1c interferes with results

Average HbA1c levels are typically higher in Hispanics, Asians, Native Americans and African Americans in comparison with Caucasians.<sup>4</sup> HbA is the primary hemoglobin present in healthy adults.<sup>1</sup> Various hemoglobin molecules exist, such as Hb C, D, E, F, S, O, G and many more. These variations of the hemoglobin molecule may lead to disease states, such as sickle cell anemia (HbS), or may remain undetected for an individual's entire lifetime.<sup>1</sup> The presence of variant hemoglobin molecules may affect the HbA1c results obtained by analyzers, specifically those using varying methodologies. This is yet another example of the need for standardization of HbA1c methodologies.<sup>6</sup>

A number of hemoglobin variants, such as Hb S, C, D, and E all influence HbA1c measurements.<sup>7</sup> The most common hemoglobin variants are HbS, HbC, HbE and HbD (in order of highest to lowest prevalence in the United States), all of which result from single amino acid substitutions in hemoglobin's  $\beta$  chain.<sup>8</sup> Physicians who treat patients with hemoglobinopathies may be unaware that a patient is heterozygous for a particular hemoglobin variant when treating patients with diabetes mellitus. It is important for healthcare providers to keep in mind that the life span of RBC affects HbA1c levels. These heterozygous patients often have normal red cell survival time and thus HbA1c values are accurate for most of these patients. Hemoglobin variants preventing glucose binding or affecting the results from a particular methodology also must be considered. Since immunoassays rely on the recognition of the structure of the first 4 to 10 amino acids on the N-terminal glycated hemoglobin S and C variants (close to the N-terminus), results can be affected. The variations in hemoglobin E and D are far enough away from the N-terminus of the  $\beta$  chain that they do not affect HbA1c results. Each of the four hemoglobin variants alters the charge of the hemoglobin molecule, leaving the possibility of erroneous results in the ion-exchange method.<sup>6</sup>

Many clinical laboratories employ immunoassay methodology. Immunoassays measure HbA1c using either polyclonal or monoclonal antibodies. The antibodies are directed toward the glycated N-terminal group on the  $\beta$  hemoglobin chain. First the total hemoglobin is determined. Then, the HbA1c is quantified and a percentage is calculated for total HbA1c. Immunoassays provide quantitative measurements of HbA1c serologically.<sup>1</sup> Increased fetal hemoglobin (HbF)

above 15%, often seen in patients with thalassemia, causes a low estimate of HbA1c. The erroneous results may lead to the inaccurate perception that the patient's diabetes is under control when in fact the patient's average blood glucose is much higher than the value HbA1c is representing. Other hemoglobin variants including HbAS and HbAC cause false increases in HbA1c measurements while HbAD and HbAE are not affected in immunoassay methodologies.<sup>6</sup>

Affinity chromatography, a type of liquid chromatography, separates molecules of interest from a solution by utilizing its specific affinity for ligands present in a matrix that the solution is run through.<sup>9</sup> In this case, affinity chromatography utilizes the principle of the attachment of glycosylated hemoglobin to the boronate group of a resin. A buffer helps to specifically elute HbA1c from the resin. The advantages of this method is that it does not depend on temperature and is generally believed to be unaffected by the common hemoglobin variants of hemoglobin F, S and C.<sup>1</sup> Recent research, however, suggests that elevated levels of hemoglobin F that often occur in conditions such as thalassemia and hereditary persistent fetal hemoglobin (HPFH) may interfere with readings in boronate affinity methods.<sup>10</sup> Boronate affinity chromatography relies on the separation of glycated hemoglobin from nonglycated hemoglobin (without concern for the hemoglobin species), so most hemoglobin variants do not interfere with the results obtained with this method. The coplanar cis-diol groups of the glucose bound to HbA1c are utilized in the boronate affinity method of HbA1c determination, as these groups interact with *m*-aminophenylboronic acid present in the reagent. Research suggests little interference from hemoglobin variants in boronate affinity chromatography.<sup>6</sup>

Ion-exchange chromatography uses a positively charged resin bed to attract negatively charged hemoglobin molecules. Glycated hemoglobin carries the most negative charge, allowing it to be eluted from the resin bed with a pH-specific buffer. This method, however, is temperature-dependent and can be affected by hemoglobinopathies.<sup>1</sup> High-pressure liquid chromatography (HPLC) is an ion-exchange chromatographic method that is useful in separating all 3 forms of glycosylated hemoglobin (A1a, A1b & A1c). Components of the blood are separated using a column with silica-based particles which allows a solvent to travel

through the column. Depending on the specific affinity of each analyte between the mobile phase and the stationary phase, each form of glycosylated hemoglobin molecules migrates up the column at different speeds, resulting in the separation of the mixture into the three forms of glycosylated hemoglobin.<sup>11</sup> HbAE and HbAD may show interference in the measurement accuracy using methods based on ion-exchange HPLC.<sup>6</sup>

Electrophoresis separates glycosylated hemoglobin molecules based on charge differences. Patient samples are loaded into a gel matrix and attached to an electricity source. Based on the slight charge differences between the various glycosylated hemoglobin molecules, the distances that various proteins travel towards the anode vary. Hemoglobin A typically comprises 95-98% of normal adult hemoglobin. Hemoglobin F should only be present in small quantities ranging from 0.8-2%. Electrophoresis produces erroneous results when hemoglobin F is in higher quantities, as the significantly elevated fetal hemoglobin levels alter the relative concentrations of hemoglobin A and ultimately the measured HbA1c levels. Electrophoresis, however, is useful in identifying hemoglobin subgroups that may interfere with HbA1c results, including hemoglobin S, C and F.<sup>12</sup>

Furthermore, isoelectric focusing (IEF) is a method of electrophoresis that relies on respective isoelectric points (pI) to isolate proteins within a specific pH gradient.<sup>13</sup> IEF testing also aids in the detection of changes in hemoglobin molecule due to post-translational modifications. Based on differences in pIs, hemoglobin variants can be separated quickly. IEF is sensitive, however, it presents the disadvantage of interference from pre-HbA1c.<sup>1</sup>

#### List of NGSP Certified Methods

Of all the methodologies certified by National Glycohemoglobin Standardization Program (NGSP), the following represents a breakdown of the frequency of the methods used in 183 testing platforms. Table 1 shows general descriptions of methodologies, while Table 2 provides a detailed breakdown of frequency of specific methodology's frequencies based on use in testing platforms alone.

#### The Need for Standardizing of HbA1c Measurements

The Diabetes Control and Complications Trial (DCCT) first demonstrated the need for control of glucose levels

in diabetes mellitus patients. Glycated hemoglobin began being reported out predominantly as HbA1c in 1999 shortly following the formation of NGSP.<sup>15</sup> NGSP works to standardize HbA1c assays.<sup>16</sup> NGSP certifies manufacturers of HbA1c instrumentation as well as manages proficiency-testing requirements to reduce variability in HbA1c results. Controversy exists about whether to report HbA1c according to NGSP units (% HbA1c) or the International Federation of Clinical Chemistry (IFCC) units (mmol/mol).<sup>15</sup>

**Table 2.** Frequency of Use of HbA1c Methodologies in Instrumentation based on NGSP Certified Methodologies<sup>14</sup>

Methodology	Frequency of Use in Instrumentation
Immunoassay	95
Ion-exchange High Pressure Liquid Chromatography	44
Boronate Affinity	13
Enzymatic	27
Capillary Electrophoresis	3
Boronate Fluorescent Quenching	1
<b>Total</b>	<b>183</b>

Due to the use of different methodologies, laboratories often obtain conflicting results. This lack of standardization leads to inconsistent results, which could affect treatment plans. Overall, there is a need for the reproducibility of HbA1c results across all laboratories. The United States, Japan and Sweden have comparison methods; however, these vary in specificity, references ranges and action limits and fail to correlate between the various geographic areas involved in the standardization efforts. The currently accepted measurement of HbA1c is a reference system utilizing metrological traceability.<sup>17</sup> Metrological traceability is the effort "to ensure comparable and portable laboratory results."<sup>18</sup> The US, Japan and Sweden have decided to use HPLCs results for comparison purposes. The various HPLC methods utilized by the aforementioned countries produced significant variances in HbA1c measurements, leading to further consensus that HbA1c methodology must be strictly regulated.<sup>17</sup>

The IFCC has established protocols to standardize HbA1c measurements. First, a standard definition of HbA1c was agreed upon. IFCC standardized HbA1c

units to mmol/mol and also began using NGSP percentages to find eAG values in mmol/liter or mg/dL. IFCC results are accuracy-based while NGSP measurements are directed toward clinical outcomes and health-related goals of diabetes mellitus patients. Correlations exist between IFCC/NGSP values, although the absolute numbers are different. A longstanding debate exists over which value to report.<sup>19</sup> The IFCC and NGSP work in different ways to help standardize HbA1c. IFCC works with manufacturers of HbA1c methodologies to ascertain they are “traceable to an accuracy base.” The IFCC does not set a limit on the degree of uncertainty between a particular manufacturer’s methodology and the IFCC values obtained. NGSP, on the other hand, does set acceptable limits to which the assay methods must adhere. These limits are based on clinical requirements.<sup>15</sup>

#### Effect of Hemoglobin Variants on HbA1c Measurements

Based on improved precision and the ability to process large volumes of samples, most laboratories use either HPLC or immunoassay for HbA1c determination.<sup>20</sup> When variant forms of hemoglobin molecules are present in a patient’s sample, newer HPLC analyzers can identify the variants and display peaks where variant forms have been resolved (as an indicator of their presence and ability to interfere with results) on a computer-generated chromatogram. Some HPLC analyzers also produce quantitative values from the given peaks. Often immunoassay analyzers are used in point-of-care testing (POCT). The immunoassay methodology is affected by variant forms of hemoglobin molecule and does not provide a visual or quantitative value notifying the user of their existence. For this reason, physicians may base treatment on inexact HbA1c levels determined by these potentially inaccurate POCT instruments.<sup>21</sup> For example, falsely low A1c measurements may inappropriately prompt the physician to “ease up” on treatment.

Inaccurate results often are reported due to the effect of various hemoglobin molecules with the type of testing methodology used. Patients most frequently affected are those with hemoglobin S seen in sickle cell trait and hemoglobin E. Additionally, patients with sickle beta thalassemia and individuals with HbF have higher than normal levels of hemoglobin F, which can falsely elevate or lower A1c values, depending on the methodology

used. To avoid these errors, hemoglobin electrophoresis may be indicated for patients with diabetes mellitus whose blood glucose and HbA1c levels are in disagreement due to differences in HbA1c methodologies used.<sup>21</sup>

Based on the methodology used, hemoglobin variants interference may be present in different forms. Immunoassay methods will not alert to hemoglobin variants, so they often are not suspected unless the HbA1c results are exceptionally high or low. This presents an obvious problem, as the hemoglobin variant will not be suspected if a reasonable (although inaccurate) value is obtained. Ion-exchange HPLC methods produce chromatograms that serve as visual alerts of possible variants. It is vital that lab personnel and physicians be trained in interpreting the chromatograms produced in order to notice unusual patterns.<sup>21</sup> Although unaffected by other variants, the boronate affinity methods are hindered by persistent hemoglobin F which may affect HbA1c results. Healthcare teams must consider patient population makeup and the likelihood of hemoglobin variants existing when selecting a method for measuring HbA1c. For example, HbF and HbA1c have similar charges, making it difficult to separate them. If HbF is present, one may experience inaccurate (often elevated) measurements using electrophoresis-based methodologies.<sup>6</sup>

HbA1c measurements often are inaccurate in patients with hemolytic diseases and hemoglobinopathies. Patients suffering from thalassemia major are at an increased risk for developing diabetes mellitus. Due to this fact, monitoring these patients’ blood glucose levels is of critical importance.<sup>22</sup> Individuals with thalassemia major have high levels of fetal hemoglobin, so measurements of HbA1c are often inaccurate. Studies show that among thalassemia major patients receiving regular blood transfusions, both HbA1c and fructosamine measurements are dependable options. In patients with substandard access to healthcare and patients not undergoing regular transfusions, fructosamine tests may be a more accurate and reliable option than HbA1c testing.<sup>23</sup>

#### Genetic Variants and Their Effect on HbA1c

HbA1c is genetically affected by differences in several loci. Genetic factors influencing the expression of genes, turnover rates of erythrocytes and the glycation of



hemoglobin molecule can lead to elevated HbA1c levels. Thus far, 10 genetic loci have been associated with variations in HbA1c levels.<sup>24</sup> Several single nucleotide polymorphisms (SNPs) have been shown to affect glucose levels in diabetic patients. In type 1 diabetes mellitus patients, 4 loci have been shown to affect HbA1c levels. These include *BNC2*, *SORCSI*, *GSC* and *WDR72*. Studies have failed to correlate these loci with glycemic levels in type 2 diabetics, although the effects of *SORCSI* have not been completely ruled out.<sup>25</sup>

**Problems with POCT**

POCT of HbA1c is increasingly utilized in physicians’ office laboratory settings for both ease and practical purposes. POCT allows for rapid results versus the use of traditional laboratory instrumentation.<sup>26</sup> POCT is convenient for use at routine doctor visits as they commonly require only a single drop of blood and can provide blood HbA1c results that are available when a physician sees a patient, often in as little as a few minutes. Rapid results are crucial to maintain timely communication and consistent dialogue between patient and physician.<sup>27</sup>

Accurate POCT of HbA1c would greatly increase the quality of care received by diabetics, as HbA1c results can be measured and received in one office visit. Unfortunately, many point-of-care analyzers do not meet the Clinical Laboratory Standard Institute’s (CLSI) quality requirements. Out of the eight currently-accepted analyzers measured, only two met nationally-accepted standards.<sup>28</sup> It is critical to know that, although useful for casual monitoring of HbA1c in physician’s offices and clinic settings, POCT HbA1c analyzers are not accurate enough for the diagnosis of diabetes mellitus.<sup>29</sup> The advantages and disadvantages of POCT testing are summarized in Table 3.

**Table 3.** Advantages & Disadvantages to HbA1c POCT Tests

Advantages	Disadvantages
Speed of Results Ease	Possible Inaccurate Results Doctor/Nurse not trained on instrument
Allows for more physician-patient interaction	

**Other Glycated Protein Testing**

Similar to their interactions with hemoglobin, molecules of glucose also join with other proteins to form stable ketoamines referred to as fructosamines. Albumin, the

most abundant serum protein, is also glycated. Glycated albumin (GA) measurements may be obtained and utilized similarly to HbA1c measurements.<sup>20</sup> HbA1c measurements are reflective of average blood glucose levels over the approximately 2-3 months, while GA measurements reflect a shorter time period of the previous 2-3 weeks.<sup>30</sup>

GA may be considered as an alternative to HbA1c measurements. The advantage of using GA measurements is in the reduced amount of time needed between measurements. This allows physicians to make more frequent adjustments to a diabetic’s treatment plan to properly manage blood glucose levels. GA and glucose measurements are useful in detecting the frequent shifts in blood glucose levels experienced in gestational diabetes. Women with gestational diabetes also benefit from the use of GA measurements. In addition, HbA1c measurements often are inaccurate in disorders that shorten RBC survival. These are cases in which GA serves as an alternative measurement to HbA1c testing. Lastly, GA levels are a better indicator of glycemic control than HbA1c in patients with hemoglobin variants. GA testing is not in widespread use. With increasing testing availability and broadening knowledge of the benefits of testing additional glycated proteins, the effectiveness of diabetes mellitus monitoring may be improved.<sup>31</sup> A summary of the advantages and disadvantages of GA testing can be seen in Table 4.

**Table 4.** Advantages and Disadvantages of Glycated Albumin Testing

Advantages	Disadvantages
Monitor blood glucose levels more frequently	Less experience with use in the United States
Reflective of a shorter period of time (2-3 weeks) to make more frequent adjustments to treatment based on recent levels	Reflective of a shorter period of time, therefore not indicative of overall blood glucose control over several months
Useful in those with decreased RBC survival rates	
Useful in those with hemoglobin variants	

Methodologies for GA determination includes affinity chromatography, colorimetric assay and immunoassays. Of these methodologies, the colorimetric assay is most frequently used. The colorimetric assay utilizes the principle that ketoamines reduce nitroblue tetrazolium to

form an insoluble compound. The change in absorbance is then measured spectrophotometrically at 525nm. Numerous automated versions of this assay exist for easy laboratory use. GA measurements are affected by samples from individuals with thyroid disease or low albumin levels. The presence of hemolysis, bilirubin or lipemia in a sample also affects the accuracy of results measured by GA methodology.<sup>20</sup>

HbA1c provides valuable information for the diagnosis and monitoring of blood glucose control in diabetic patients. Various methodologies exist for the measurement of HbA1c, each offering individual benefits and drawbacks. Patients with hemolytic diseases and variant hemoglobinopathies can be affected by inaccurate results.<sup>22</sup> The standardization of methodologies across laboratories to provide reproducible and reliable results is crucial in providing proper patient care.

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