THE STUDY OF PROPERTIES AND FACTORS AFFECTING THE COMMUTABILITY OF POOLED BLOOD MATERIAL FOR HBA_{1C} MEASUREMENTS

KANOKWAN NGUEANCHANTHONG

A Thesis Submitted to the Graduate School of Naresuan University

in Partial Fulfillment of the Requirements

for the Master of Science in Biomedical Sciences - (Type A 1)

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Thesis entitled " The study of properties and factors affecting the commutability of pooled blood material for HbA_{1C} measurements."

by Kanokwan Ngueanchanthong

has been approved by the Graduate School as partial fulfillment of the requirements for the Master of Science in Biomedical Sciences - (Type A 1) of Naresuan University

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Title THE STUDY OF PROPERTIES AND FACTORS AFFECTING THE COMMUTABILITY OF POOLED BLOOD MATERIAL FOR HBA_{1C} MEASUREMENTS

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ABSTRACT

Measurement of hemoglobin A_{1C} (HbA_{1C}) is essential for the diagnosis and treatment of diabetes mellitus (DM). Processed blood materials (PBMs), such as frozen pooled blood (FPB), are commonly used for External Quality Assessment (EQA). However, their commutability may be affected by factors such as hemolysis and matrix modification. This study evaluated the commutability of FPB and identified the factors influencing their performance in HbA_{1C} measurements. HbA_{1C} contents in 24 clinical blood samples (CBSs) and 10 PBMs, including FPBs, single-donor blood (SDB), and *in vitro* glycated blood (IGB), were analyzed using enzymatic (EN), capillary electrophoresis (CE), cation-exchange high-performance liquid chromatography (CE-HPLC), turbidimetric immunoassay (TI), and boronate affinity HPLC (BA-HPLC). EN served as the reference measurement procedure. Commutability was assessed using Deming regression and 95 prediction intervals. The hematocrit (HCT), visual appearance, plasma absorbance (PA), hemolysis index (HI), pH, and debris cells (DC) were analyzed. FPB1 and SDB were commutable across all measurement procedures. FPB2 and FPB4 were commutable with CE, CE-HPLC, and TI but non-commutable with BA-HPLC when HbA_{1C} was ≥6.23%, as data points fell outside the tolerance limits of the Deming regression lines. Non-commutable PBMs showed higher PA and HI along with lower pH. DC affected HbA_{1C} quantification

using CE-HPLC. Hemolysis, as assessed by PA and HI, affects FPB commutability. These factors should be considered in EQA programs, particularly for BA-HPLC. HCT and DC did not directly affect commutability; however, maintaining appropriate HCT levels and monitoring DC-related chromatographic interference are crucial for ensuring FPB reliability in HbA_{1C} comparisons.

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CHAPTER I

INTRODUCTION

Background and rationale

Diabetes mellitus (DM) is a major non-communicable disease (NCD) and a growing global public health concern. According to the International Diabetes Federation (IDF), 537 million adults had DM in 2023, projected to rise to 643 million by 2030 (Atlas, 2023). In Thailand, the Ministry of Public Health reported 3.3 million cases and 16,388 deaths in 2020 (Ministry of Public Health, 2023). Complications from poor blood sugar control affect various organs, emphasizing the need for accurate blood sugar measurement.

Hemoglobin A_{1C} (HbA_{1C}) or glycated hemoglobin (Hb) is a measurement of a compound formed by the binding of glucose to the valine amino acid at the N-terminal end of the beta chain of Hb in red blood cells (RBCs), which have a lifespan of approximately 2 -3 months. Therefore, measuring HbA_{1C} can provide an average measurement of accumulated glucose levels over a period. Since 2009, experts from the American Diabetes Association (ADA) have recommended using HbA_{1C} content for diagnosing and monitoring the treatment of diabetes (DM) patients (AmericanDiabetesAssociation). The diagnostic criterion for DM is an HbA_{1C} content of $\geq$ 6.5%, and HbA_{1C} content between 5.7% and 6.4% indicates a high-risk group for developing DM (Pre-diabetes mellitus) (AmericanDiabetesAssociation, 2021). The treatment target for DM is to maintain HbA_{1C} content below 7% consistently, which can lead to effective treatment and reduce complications of DM (AmericanDiabetesAssociation, 2021; DiabetesAssociationofThailand, 2018).

In Thailand, HbA_{1C} testing has become increasingly popular because it does not require fasting. However, it should be performed in laboratories that adhere to the National Glycohemoglobin Standard Program (NGSP) or use the Diabetes Control and Complications Trial Reference Assay (DCCT) as a standard method (DiabetesAssociationofThailand, 2018). HbA_{1C} testing techniques vary, including 1) analysis based on differences in net charge according to the type of Hb, such as cation-exchange high-performance liquid chromatography (CE-HPLC); 2) analysis

based on structural differences in Hb, such as boronate affinity HPLC (BA-HPLC) and turbidimetric immunoassay (TI); and 3) chemical reactivity, such as enzymatic assay (EN) (Sacks, 2012; V., 2016; Wongsri, 2018). Standardization and quality control are critical for ensuring the accuracy of $HbA1_C$ measurements (DiabetesAssociationofThailand, 2018).

The International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) developed reference methods for HbA_{1C} standardization, establishing a network of laboratories. Using pure HbA_{1C}, the IFCC implemented two reference methods: mass spectrometry and CE (NGSP, 2010). Additionally, target values for blood materials were determined using the IFCC secondary reference measurement procedures (IFCC Secondary RMP). These target values are critical for evaluating laboratory test accuracy.

In laboratory testing, blood materials (BMs) are essential for Internal Quality Control (IQC) programs and External Quality Assessment (EQA). Blood materials for quality control should comply with ISO 17034: General Requirements for the Competence of Reference Material Producers. This ISO standard mandates that blood materials be both homogeneous and stable before use. Additionally, materials used for measurement comparison must demonstrate commutability across different measurement methods, similar to that of clinical blood samples (CBSs) (Trapmann, Botha, Linsinger, Mac Curtain, & Emons, 2017).

At the Asia-Pacific Federation for Clinical Biochemistry and Laboratory Medicine Congress (APFCB 2024), Chris Farrell emphasized commutability's importance. He cited studies by the Royal College of Pathologists of Australasia Quality Assurance Programs (RCPAQAP), which found that lyophilized materials were non-commutable when comparing CE and TI procedure but were commutable between CE and CE-HPLC procedure. Based on these findings, the study recommended the use of fresh whole blood (FWB) for the evaluation of test result accuracy.

Fresh whole blood is highly commutable but has production limitations. Thus, processed blood materials (PBMs) offer a practical alternative. In Thailand, PBMs are prepared using methods such as frozen pooled blood (FPB), single-donor blood (SDB), and *in vitro* glycated blood (IGB). While FPB enables long-term storage,

freezing may cause red blood cell (RBC) lysis. Nevertheless, FPB materials are employed in EQA programs by the Department of Medical Sciences, Ministry of Public Health, Thailand (8). SDB and IGB materials have also been developed for EQA programs at WE Med Lab Center Co., Ltd (Lab, 2024). PBMs must retain matrix properties comparable to CBSs; however, modifications such as pooling, freezing, or additives can influence commutability (Miller et al., 2018). This study aims to assess the commutability of HbA_{1C} in PBMs and identify factors affecting commutability, ultimately optimizing PBM preparation for quality control in HbA_{1C} measurement. The objective is to improve alignment with clinical laboratory standards, thereby enhancing the reliability and accuracy of HbA_{1C} testing.

Research aims

1. To identify the factors that influence the commutability of BMs.
2. To improve the preparation procedure of BMs, ensuring their commutability and compatibility with all measurement principles.
3. To assess the homogeneity and stability of BMs.

Scope of research

This study focuses on developing a procedure to prepare BMs that closely mimic CBSs. We evaluated PBMs derived from FPBs and other PBMs, such as SBD, and unprocessed native blood samples derived from FWBs in a prospective cohort study.

The HbA_{1C} content in four commutable FPBs, measured by EN, was traceable to the IFCC target values. A total of 24 CBSs and 10 BMs were analyzed for HbA_{1C} content using five measurement procedures: EN, CE, CE-HPLC, TI, and BA-HPLC. Additionally, hemoglobin typing of CBSs and HbA_{1C} blood materials was performed using the CE procedure to assess blood types.

The commutability of blood materials was evaluated following the CLSI EP14-A4 guidelines, and non-commutable PBMs were compared with commutable PBMs to identify key factors influencing HbA_{1C} measurements (**Figure 1**).

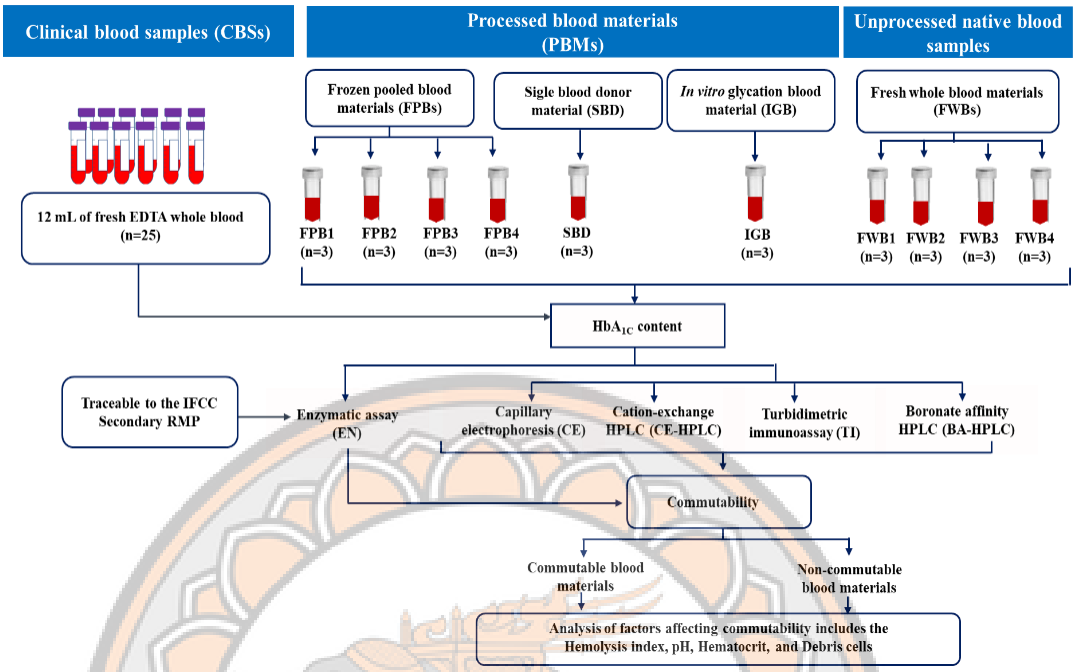


Figure 1. The scope of this study includes the preparation, characterization, and evaluation of blood materials (PBMs and unprocessed native blood samples), focusing on their commutability and factors influencing HbA_{1C} measurement accuracy

Statement of the problems

The accurate and precise measurement of HbA_{1C} is crucial for the diagnosis and management of diabetes. However, the current techniques for HbA_{1C} measurement have limitations and result in variations in the obtained results. Therefore, there is a need for reference materials (RMs) that mimic human samples and can be used for quality control (QC) and proficiency testing (PT) of HbA_{1C} measurement.

Blood materials are commonly used as biological material for preparing blood materials. However, using blood has limitations in terms of quantity and stability. Various procedures have been developed to address these issues and enable mass production and improved stability.

One such procedure involves creating pooled blood samples from remnant blood samples, which allows for the generation of materials with high HbA_{1C} content. Phanthira Wongsri (2018) studies have demonstrated that pooled blood samples can

be stored at temperatures between 2-8°C for 15-30 days (Wongsri, 2018). Furthermore, Suparak et al. (2022) have investigated the stability of frozen pooled blood samples and found that they can be stored at -70°C for up to 1 year (Suparak et al., 2022). However, it is important to consider that the freezing process may impact the biological components of red blood cells (RBCs) and potentially affect HbA_{1C} measurement in certain analysis techniques.

Another approach involves using blood donor samples to prepare blood materials. However, finding blood donors with a high content of HbA_{1C} is rare. *In vitro* glycation of blood donor samples has been used to produce BMs with the desired HbA_{1C} content (W Duanginta et al., 2023).

Each of these procedures of blood modification has its own advantages and disadvantages. Blood materials that are modified from clinical samples have inherent differences for each procedure and may potentially interfere with HbA_{1C} measurement. Hence, it is important to study the commutability of blood materials in order to identify factors that can impact HbA_{1C} measurement. Blood materials are used for measuring HbA_{1C} using various measurement procedures such as EN, CE, CE-HPLC, TI, and BA-HPLC. Therefore, non-commutable BMs need to be adjusted for parameters that can impact HbA_{1C} measurement in order to develop commutable blood materials that can be used with all measurement procedures.

Preliminary agreement

1. Diagnosis of diabetes based on HbA_{1C} content

Hemoglobin A_{1C} is a diagnostic tool used to determine if a person has diabetes. It measures the average blood sugar levels over a period of 2-3 months. The American Diabetes Association (ADA) recognizes HbA_{1C} as a diagnostic test for diabetes. According to ADA guidelines, an HbA_{1C} content of 6.5% or higher on two separate tests indicates diabetes. This means that if a person's HbA_{1C} content remains consistently at or above 6.5%, they are considered to have diabetes (AmericanDiabetesAssociation). Confirmatory testing is recommended to ensure accuracy unless the person shows clear symptoms of high blood sugar or has a random plasma glucose level of 200 mg/dL (11.1 mmol/L) or higher

(AmericanDiabetesAssociation). It's important to consider other factors such as symptoms and additional tests to confirm the diagnosis of diabetes using HbA_{1C}.

2. Quality assurance programs

Quality Assurance Programs (QAPs) are systematic processes designed to ensure the accuracy, reliability, and consistency of laboratory testing. These programs play a critical role in providing confidence in the reliability of test results. QAPs typically involve external proficiency testing, also known as EQA and IQC programs.

External quality assessment programs are managed by external organizations that provide participating labs with samples of known values. After analyzing these samples, the laboratories report their findings to the program organizers. The performance of each laboratory is assessed by the program through a comparison of the reported results with the established values. EQA should be conducted under the guidelines for proficiency testing outlined in ISO 17043 (Conformity assessment - General requirements for testing).

Internal quality control refers to the practices used in a laboratory to keep track of the precision and accuracy of the tests they conduct. This entails analyzing the outcomes after running patient samples alongside control samples with known values. IQC enables labs to find and fix any problems or variations in their testing procedures, ensuring accurate and consistent results.

3. Blood materials

Blood material is widely utilized as a sample for HbA_{1C} measurement. It encompasses all the components of blood, including red blood cells, white blood cells, platelets, and plasma. Blood material has been developed for use both domestically and internationally to measure HbA_{1C} as follows:

Suparak et al. 2022 utilized frozen pooled blood materials obtained from remnant untainted ethylenediaminetetraacetic acid (EDTA) samples to assess laboratory proficiency in Thailand since 2016. The study assessed the properties of these materials following ISO 13528 (Statistical procedures for use in proficiency testing by interlaboratory comparison) and observed that they demonstrated uniformity and stability when stored at 2-8°C for a minimum of two weeks and at -70°C for one year (Suparak et al., 2022).

The European HbA_{1C} trial was established by the IFCC to investigate the efficacy of HbA_{1C} assays. The IFCC reference measurement procedure is utilized to assign values from five approved IFCC network laboratories to three types of blood: fresh whole blood, frozen whole blood, and lyophilized hemolysate. According to a study on the stability of the test materials, fresh whole blood can be stored for up to 5 days at room temperature and up to 8 days in the refrigerator. Frozen whole blood and lyophilized hemolysate were stored in freezers at -20°C and -70°C, respectively, and were measured after 49 months (IFCC, 2021).

In 2018, Phanthira Wongsri conducted a study on the preparation of quality control materials for measuring HbA_{1C}. The study utilized test materials derived from remnant untainted blood EDTA samples. The blood samples were washed with normal saline solution (NSS) and supplemented with citrate-phosphate-dextrose-adenine (CPDA1) to achieve a hematocrit level of 40 percent. The materials were used without undergoing freezing. The study demonstrated that the materials, categorized into three levels of HbA_{1C} (Level 1: 4.0-6.4, Level 2: 6.5-9.9, Level 3: 10.0-14.0), consistently exhibited homogeneity and stability when stored at temperatures of 2-6°C for 15, 30, and 15 days, respectively (Wongsri, 2018).

In order to prepare blood samples for HbA_{1C} with the desired high HbA_{1C} content that would be compatible with two immunoassays, Duanginta et al., 2023 conducted a study to identify suitable conditions for in vitro glycation. The study findings revealed that incubating erythrocytes with 400 mM D-glucose for 15 hours at 37°C resulted in suitable *in vitro* glycation conditions, producing HbA_{1C} materials with intact and sufficiently stable erythrocytes. Two of the blood materials, namely GLY-01 (percent of HbA_{1C} 8.7%, 70 mmol/mol) and GLY-02 (percent of HbA_{1C} 6.6%, 48 mmol/mol), demonstrated commutability between enzymatic and turbidimetric immunoassays. However, GLY-03 (percent of HbA_{1C} 7.9%, 61 mmol/mol) showed non-commutability. The study also observed the dissolution of red blood cells in GLY-03, which could be attributed to errors in the material preparation process (W Duanginta et al., 2023).

Research hypothesis

1. Blood materials can be assessed for commutability when comparing paired difference measurement procedures.
2. Factors influencing the measurement of non-commutable FPBs can be identified and used to refine preparation procedures, ensuring that PBMs are commutable and suitable for laboratory performance evaluation.

CHAPTER II

LITERATURE REVIEW

1. Diabetes mellitus (DM)

A chronic metabolic disorder called diabetes mellitus (DM) is characterized by hyperglycaemia, or high blood sugar. It happens when the body is either unable to use the insulin it produces or does not produce enough of it. The pancreas secretes the hormone insulin, which controls how proteins, fats, and carbohydrates are metabolized and aids in the maintenance of normal blood sugar levels. There are various types of DM, including (AmericanDiabetesAssociation, 2021):

Type 1 DM: This form of DM is an autoimmune disorder in which the body's defense mechanisms attack and kill the insulin-producing cells in the pancreas.

Type 2 DM: The most common type of DM is type 2 DM. It typically occurs when the body develops insulin resistance or does not produce enough insulin to maintain proper blood sugar levels.

Gestational DM: Gestational DM usually goes away after childbirth and develops during pregnancy. When the body is pregnant, the demand for insulin rises, and the body is unable to meet that demand. Type 2 DM is more likely to strike women who have gestational DM in the future.

Other Types: There are other specific types of DM, such as genetic forms of diabetes (e.g., diabetes that starts during puberty) and DM caused by certain medications or medical conditions.

Diagnosing DM involves a thorough process that includes a medical history assessment, physical examination, and specific laboratory tests. The identification of symptoms, family history, and risk factors is aided by medical history. A physical examination searches for DM-related symptoms and signs. To confirm the diagnosis, lab tests like the fasting plasma glucose (FPG) test, oral glucose tolerance test (OGTT), and HbA_{1C} test are used (AmericanDiabetesAssociation; DiabetesAssociationofThailand, 2018). The FPG test measures blood sugar levels after a fast, and DM is diagnosed when the result is 126 mg/dL or higher on two separate

occasions. A result of 200 mg/dL or higher after two hours in the OGTT indicates DM (DiabetesAssociationofThailand, 2018). The test measures blood sugar levels before and after consuming a sugary drink. The HbA_{1C} test measures the average blood sugar level over the previous few months; DM is indicated if the A_{1C} content is 6.5% or higher on two tests.

Hemoglobin A_{1C} is a useful tool because it does not require fasting and provides a reliable indication of long-term blood sugar control. It is especially beneficial in assessing how well DM management strategies, such as diet, exercise, and medication, are working overtime. In individuals with DM, Hemoglobin A_{1C} content is typically higher than normal, reflecting poor blood sugar control. The test calculates the proportion of hemoglobin, a protein found in red blood cells (RBCs), that is attached to glucose. Higher HbA_{1C} content is a result of more glucose attaching to hemoglobin as blood sugar levels rise.

The American Diabetes Association (ADA) advises people with DM to work toward maintaining a HbA_{1C} content under 7% to lower their risk of complications from the disease (AmericanDiabetesAssociation). Target levels, however, may differ based on unique circumstances, and healthcare professionals may establish specific objectives based on elements like age, general health, and the presence of other medical conditions.

Hemoglobin A_{1C} content is regularly checked to help people with DM and their healthcare providers track the efficacy of treatment regimens, make necessary medication or lifestyle changes, and reduce the risk of complications brought on by poorly controlled blood sugar levels. It is important to note that HbA_{1C} content is not a substitute for daily self-monitoring of blood sugar levels. Regular blood sugar testing, along with HbA_{1C} measurements, provides a comprehensive view of blood sugar control and guides diabetes management strategies. Consulting with a healthcare professional is crucial for interpreting HbA_{1C} results and developing an individualized DM management plan.

2. Whole blood sample

The whole blood sample (WBCs) is composed of various components, including red blood cells, white blood cells, platelets, and plasma (**Figure 2**).

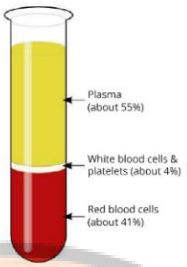


Figure 2 Blood composition after centrifugation separation (David Warmflash, 2016)

1. **Red blood cells (RBCs):** These cells are the most abundant in the blood and are responsible for delivering oxygen to the body's tissues. Hemoglobin, a protein found in RBCs, binds to oxygen, giving blood its characteristic red color.
2. **White blood cells (WBCs):** These cells are crucial components of the immune system, playing a vital role in defending the body against illnesses and infections. White blood cells come in various forms, including neutrophils, lymphocytes, monocytes, eosinophils, and basophils, each with a specific function in the immune response.
3. **Platelets:** Small cell fragments called platelets are essential to the clotting process. In order to stop excessive bleeding, platelets cling to the site of the injury and form a clot.
4. **Plasma:** Plasma acts as the liquid component of blood. This accounts for approximately 55% of the total blood volume. It is a yellow liquid that transports many substances. Includes hormones, nutrients, waste products, antibodies, and clotting factors. Plasma plays an important role in regulating body temperature and helping maintain blood pressure.

When a whole blood sample is collected for laboratory testing, it is typically processed to separate the different components. This allows for specific analysis of the individual components, such as measuring HbA_{1C} content for diabetes monitoring or assessing blood cell counts for various medical purposes.

3. Anticoagulant

An anticoagulant is a substance that prevents the clotting or coagulation of blood. It is commonly used in medical and laboratory settings to maintain blood in a liquid state, preventing the formation of clots. There are several types of anticoagulants available, including:

1. Heparin: Heparin is a commonly used anticoagulant that is derived from animal sources, such as porcine intestine or bovine lung. It functions by increasing the activity of antithrombin III, which renders clotting factors like thrombin and factor inactive. Heparin is often used in clinical settings, such as during surgeries, dialysis, or to prevent blood clotting in medical devices like catheters.

2. EDTA (Ethylenediaminetetraacetic acid): Ethylene diaminetetraacetic acid is primarily used as an anticoagulant in laboratory settings for blood collection and sample preparation. It works by binding calcium ions, which are essential for the coagulation cascade, thereby inhibiting the clotting process. EDTA is commonly used in hematology and blood banking laboratories for tests such as complete blood count (CBC) and blood typing respectively.

3. CPDA1 (Citrate-Phosphate-Dextrose-Adenine): Citrate-phosphate-dextrose-adenine is an anticoagulant solution used for blood storage and preservation in blood banks. It contains citrate to bind calcium ions and prevent clotting, phosphate as a pH buffer, dextrose as an energy source for RBCs, and adenine to support cell metabolism. Citrate-phosphate-dextrose-adenine is commonly used for the long-term storage of donated blood for transfusion purposes (d'Almeida et al., 2000).

4. Red blood cells (RBCs)

The most various cells within the circulatory system are ruddy blood cells, or erythrocytes. They are fundamental for moving squander items like carbon dioxide from the body's tissues back to the lungs for excretion as well as oxygen from the lungs to the body's tissues. Red blood cells have a unique structure that is optimized for their function:

- **Shape:** They are biconcave disc-shaped cells, which increase their surface area and allow for efficient gas exchange. This shape encourages the fast dissemination of oxygen and carbon dioxide over their films.

- **Lack of nucleus:** Develop RBCs don't have a core. This nonattendance of a core gives more space for hemoglobin, the oxygen-carrying protein, and permits RBCs to oblige a bigger sum of hemoglobin, improving their capacity to carry oxygen.
- **Flexibility:** Red blood cells are highly flexible and can change their shape to squeeze through narrow blood vessels, including the smallest capillaries. This adaptability empowers them to reach all parts of the body and convey oxygen to tissues in require.

These specialized features of RBCs contribute to their efficient oxygen-carrying capability and effective gas exchange within the body, supporting overall physiological functions.

5. Hemoglobin

Hemoglobin (Hb) is a protein molecule found within RBCs, and its primary function is to bind and transport oxygen and carbon dioxide. Hemoglobin consists of four protein chains: two alpha chains and two beta chains, each of which is associated with a heme group (**Figure 3**). The heme group contains an iron molecule that enables hemoglobin to bind with oxygen molecules.

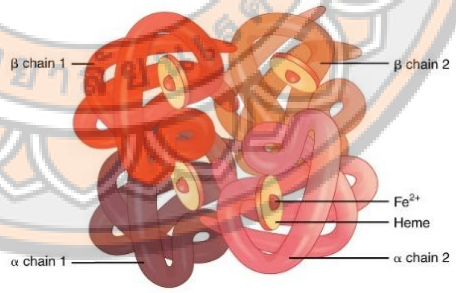


Figure 3 Four protein chains on the heme of the Hb molecule

There are different types of hemoglobin, including:

1. Hemoglobin A (HbA): Hemoglobin A is the most common and predominant type of hemoglobin in adult humans and accounts for approximately 95-98% of the total hemoglobin in the blood. It consists of two alpha chains and two beta chains.

2. Hemoglobin A_2 (HbA_2): Hemoglobin A_2 is a minor component of adult hemoglobin, accounting for about 2-3% of total hemoglobin. It is composed of

two alpha chains and two delta chains. The measurement of HbA_2 can be useful in diagnosing certain types of thalassemia, a genetic disorder affecting the production of hemoglobin.

3. Hemoglobin F (HbF): Hemoglobin F in adults typically makes up less than 1% of total hemoglobin in the normal range. Two gamma chains and two alpha chains make up its structure. After birth, Hemoglobin F gradually declines and is replaced by HbA.

4. Glycated hemoglobin (HbA_{1C}): Hemoglobin A_{1C} is a form of hemoglobin that has glucose molecules attached to it. It is used as a marker for long-term glucose control in individuals with diabetes, as mentioned earlier.

These different types of hemoglobin have specific roles and can provide important information about an individual's health and medical conditions. Testing and analyzing the levels of these hemoglobin variants can aid in the diagnosis and management of various blood disorders and diabetes.

6. Hemoglobin typing

Hemoglobin typing refers to the process of identifying and characterizing the different types of hemoglobin present in an individual's blood. Hemoglobin could be a protein found in ruddy blood cells that's capable of carrying oxygen all through the body. There are several methods commonly used for hemoglobin typing, each with their advantages and limitations. Here are some of the commonly employed methods:

1. Electrophoresis: This method separates different hemoglobin types based on their charge and size differences. Hemoglobin variants migrate at different rates in an electric field, allowing for their identification. Examples of electrophoresis methods include cellulose acetate electrophoresis (CAE), citrate agar gel electrophoresis (CAGE) and capillary zone electrophoresis (CZE).

Capillary zone electrophoresis is a specific type of electrophoresis used for hemoglobin typing. In CZE, a small amount of the patient's blood sample is placed into a capillary tube, and an electric current is applied. The different hemoglobin molecules present in the sample move at different speeds through the capillary based on their charge and size. This movement leads to their separation and allows for their identification. CZE offers advantages such as sensitivity, speed, automation, accuracy,

and quantification. It is a widely used method in clinical laboratories for hemoglobin typing and plays a crucial role in diagnosing and managing various hemoglobin disorders.

2. **High-performance liquid chromatography (HPLC):** High-performance liquid chromatography is a widely used method for hemoglobin typing. It separates hemoglobin variants based on their differences in retention time on a chromatographic column. The different peaks correspond to specific hemoglobin types, allowing for their identification and quantification.

3. **Isoelectric focusing (IEF):** 3. Isoelectric focusing separates different hemoglobin variants according to their isoelectric points, which are the pH levels at which they carry no net electrical charge. Depending on their isoelectric points, hemoglobin variants migrate to distinct positions within a gel or capillary tube, facilitating their identification.

4. **DNA-based analysis:** Genetic methods, such as polymerase chain reaction (PCR) and DNA sequencing, can be utilized to identify specific genetic changes linked to hemoglobin variants. These methods provide accurate and conclusive identification of hemoglobin types at the molecular level.

5. **Mass spectrometry:** Mass spectrometry is an advanced analytical method that can be used for hemoglobin typing. It measures the mass-to-charge ratio of hemoglobin molecules, allowing for the identification of different hemoglobin variants based on their unique mass spectrometry profiles.

The choice of hemoglobin typing method depends on factors such as availability, sensitivity, specificity, and the specific objectives of the analysis. In many cases, a combination of different methods is used to obtain a comprehensive and accurate hemoglobin typing result.

7. Glycation hemoglobin

Glycated hemoglobin, often referred to as HbA_{1C}, is a measurement of compounds resulting from the reaction between glucose and amino groups on the hemoglobin molecule, leading to the formation of ketamine. The temperature at which glycation of hemoglobin occurs is typically at normal body temperature, around 37°C or 98.6°F. The glucose molecule becomes attached to the N-terminal valines of the beta-polypeptide chains in normal adult hemoglobin (**Figure 4**). Consequently, HbA_{1C} is

reflective of blood glucose concentrations because the average lifespan of RBCs is approximately 120 days, which is also the duration during which hemoglobin can be exposed to sugar in the 2 to 3 months leading up to the test (Freeman, 2014).

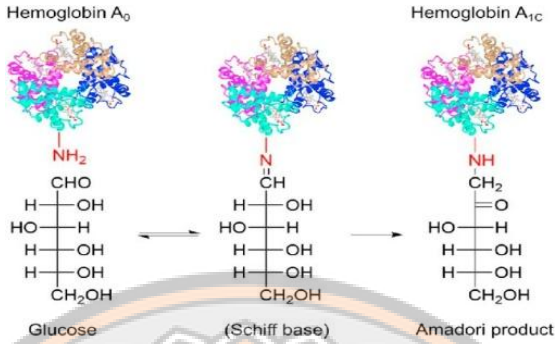


Figure 4 The manner in which glycated hemoglobin binds to the N-terminal valine of hemoglobin (Hörber, Achenbach, Schleicher, & Peter, 2020).

The HbA_{1C} test quantifies the percentage of glycated hemoglobin in the bloodstream. Glycated hemoglobin is created when glucose molecules in the blood bind to hemoglobin molecules. The HbA_{1C} content is directly impacted by the average blood glucose concentration over a period of time. When blood glucose levels remain consistently high, more glucose molecules attach to hemoglobin, leading to elevated HbA_{1C} content. Conversely, better blood glucose control results in lower HbA_{1C} content. This underscores the significance of HbA_{1C} as a valuable tool for evaluating long-term glucose management.

8. Organization for standardizing measurement of HbA_{1C}

The National Glycohemoglobin Standardization Program (NGSP) is the primary organization responsible for standardizing the measurement of HbA_{1C} in the United States. The NGSP works to ensure consistency and accuracy in HbA_{1C} testing across different laboratories and methods (**Table 1**). The NGSP collaborates with manufacturers, clinical laboratories, and professional organizations to develop and implement standardized methods for HbA_{1C} measurement. They provide reference materials and guidelines for calibration, quality control, and proficiency testing to ensure reliable and comparable results.

Table 1 List of NGSP certified laboratories in Thailand (NGSP, 2023)

Laboratory	Method/s	Certification Type	Date Certified
Clinical Chemistry and Immunology Department of Central Laboratory and Blood Bank, Faculty of Medicine, Vajira Hospital, Navamindradhiraj University, Bangkok,Thailand	Roche Cobas c 513	Level II Laboratory	February, 2023
Clinical Chemistry, Thammasat University Hospital, Pathumthani, Thailand	Arkray HA-8180V	Level II Laboratory	February, 2023
Clinical Chemistry, Diagnostic Laboratory, MaharajnakornChiangmai Hospital, Faculty of Medicine Chiangmai University, Chiangmai, Thailand	Roche Cobas 8000 (c502)	Level II Laboratory	February, 2023
Clinical Immunology and Chemistry Laboratory Unit, Clinical Laboratory Section, Srinagarind Hospital, KhonKaen University, KhonKaen, Thailand	Roche Cobas c 513	Level II Laboratory	February, 2023
Biochemistry Division, Department of Clinical Pathology, Phramongkutklao Hospital, Bangkok, Thailand	Roche Cobas c 513	Level II Laboratory	January, 2023
Hematology and Microscopy Laboratory, Bhumibol Adulyadej Hospital, Bangkok, Thailand	Roche Cobas c 513	Level II Laboratory	January, 2023

Laboratory	Method/s	Certification Type	Date Certified
Clinical Chemistry Laboratory, Biochemistry Department, Police General Hospital, Bangkok, Thailand	Roche Cobas c 513	Level II Laboratory	January, 2023
Siriraj Hospital-Mahidol University, Bangkok, Thailand	Roche Cobas c 513 Tosoh G11	Level I Laboratory	October, 2022
Immunology Laboratory, Ramathibodihospital-Mahidol University, Bangkok, Thailand	Roche Cobas c513	Level I Laboratory	July, 2022
Immunology Laboratory, Ramathibodihospital-Mahidol University, Bangkok, Thailand	Abbott Alinity c	Level I Laboratory	July, 2022
Ramadhibodi Chakri Naruebodindra Hospital, Samut Prakan, Thailand	Abbott Alinity c	Level II Laboratory	July, 2022
Clinical Chemistry, Rajavithi Hospital, Bangkok, Thailand	Abbott Alinity c	Level I Laboratory	June, 2022

Another important organization in the standardization of HbA_{1C} measurement is the IFCC. The IFCC established the reference measurement procedure (RMP) for HbA_{1C} to serve as an international standard. This procedure enables traceability of HbA_{1C} results to a common reference system, ensuring harmonization and comparability of measurements worldwide (**Figure 5**).

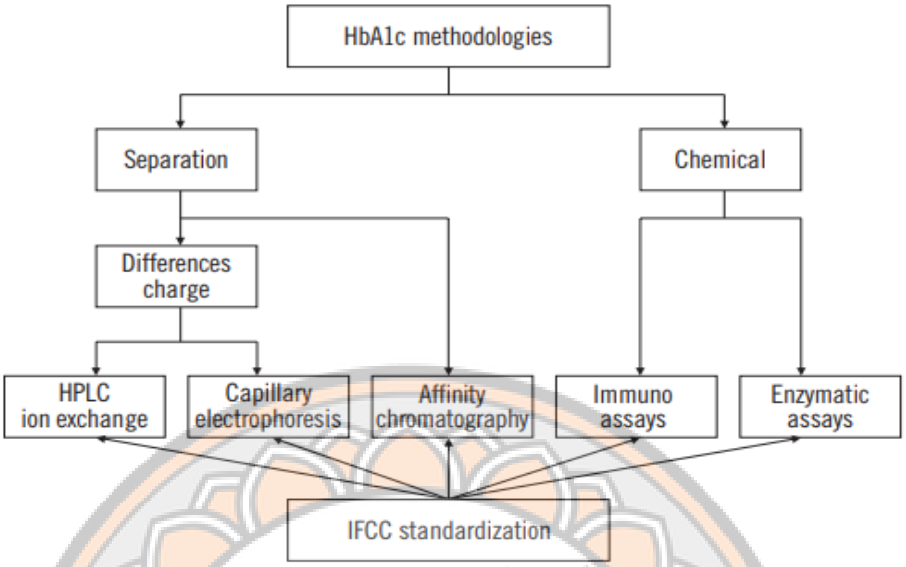


Figure 5 HbA_{1C} measurement concepts and their analytical traceability to the IFCC-RMP (Weykamp, 2013).

The NGSP and IFCC have collaborated to align their efforts and promote standardized HbA_{1C} reporting using the NGSP-derived units (eAG) and IFCC-derived units (mmol/mol). This collaboration helps improve the consistency and interpretation of HbA_{1C} results globally. The consistency and interpretation of HbA_{1C} results are improved globally as a result of this collaboration. A master equation (NGSP = [0.09148 * IFCC] + 2.152) has been developed to represent the relationship between the HbA_{1C} results from the NGSP network (percent of HbA_{1C}) and the IFCC network (mmol/mol) (**Table 2**) (NGSP). This relationship will be closely monitored, and any alterations will be investigated. The NGSP certification process will remain unchanged, and, of greater significance, test results for NGSP-certified methods will continue to be directly traceable to both the Diabetes Control and Complications Trial (DCCT) reference and the IFCC reference in the future.

Table 2 Convert between NGSP, IFCC and eAG (NGSP)

NGSP HbA_{1C} (%)	IFCC HbA_{1C} (mmol/mol)	eAG (mg/dL)	eAG (mmol/l)
5.0	31	97	5.4
6.0	42	126	7.0
7.0	53	154	8.6
8.0	64	183	10.2
9.0	75	212	11.8
10.0	86	240	13.3
11.0	97	269	14.9
12.0	108	298	16.6

Together, the NGSP and IFCC work to establish and maintain standardized methods, reference materials, and reporting systems for HbA_{1C} measurement, facilitating accurate diabetes diagnosis, management, and research.

9. HbA_{1C} measurement

The HbA_{1C} measurement is performed by taking a blood sample, usually obtained through a simple fingerstick or venous blood draw. The blood sample is then sent to a laboratory for analysis. Currently, the techniques for HbA_{1C} measurement are several techniques, each with different techniques of detection, such as charge difference measurement, structural difference measurement, and chemical reaction measurement. The most common techniques used are as follows:

1) Turbidimetric immunoassay

Turbidimetric immunoassay is a widely used method for measuring HbA_{1C} content in clinical laboratories. This technique relies on the specific binding between antibodies and antigens to detect and quantify the target analyte. HbA_{1C} refers specifically to the glycated form of hemoglobin, where glucose molecules are attached to the N-terminal glycine residues of the Hb ß-chain. In HbA_{1C} immunoassays, antibodies are developed to recognize and bind to the glycated amino acids at the N-terminal end of the Hb ß- chain. These antibodies are carefully selected and designed to have high specificity for the glycated amino acids. They can

differentiate between glycated and non-glycated forms of hemoglobin. By targeting the N-terminal glycated amino acids, the antibodies ensure that they specifically bind to HbA_{1C} and not to other forms of hemoglobin. The binding of the antibodies to the glycated amino acids forms an immune complex, which can be detected and quantified using various detection methods (**Figure 6**). The intensity of the detected signal is directly proportional to the amount of HbA_{1C} present in the sample, enabling the determination of HbA_{1C} content.

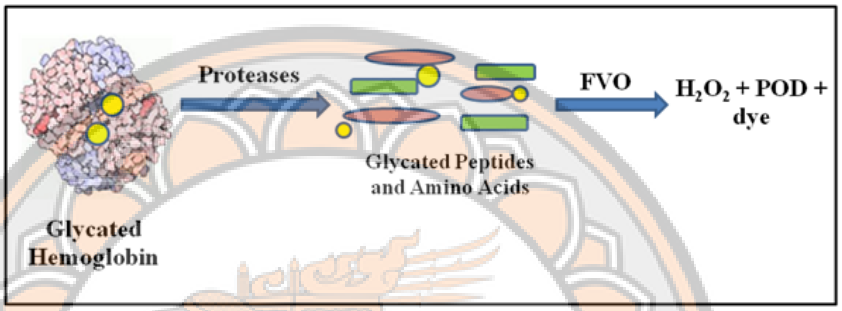


Figure 6 Latex enhanced immunoassay method (Gupta, Jain, & Chauhan, 2017).

2) Enzymatic assay

Enzymatic assays are used to measure the amount of glycated hemoglobin (HbA_{1C}) in the blood. In the enzymatic assay, a specific enzyme, such as fructosyl peptide oxidase (FPO), is used to catalyze the degradation of the glycated hemoglobin molecule. FPO specifically acts on the glycated N-terminal amino acids of the Hb ß-chain, breaking the glycation bonds and releasing fructosyl amino acids. After the enzymatic reaction, the released fructosyl amino acids can be detected and quantified using various detection techniques, such as colorimetric or spectrophotometric methods (Weykamp, 2013) (**Figure 7**). The intensity of the detected signal is directly proportional to the amount of HbA_{1C} present in the sample, allowing for the determination of HbA_{1C} content.

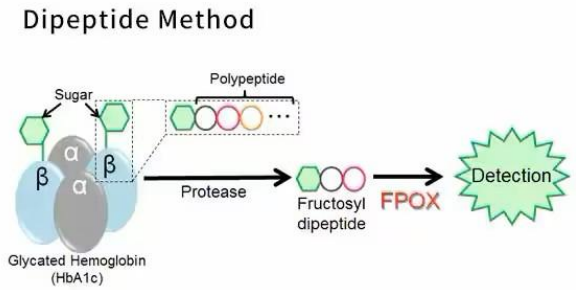


Figure 7 The measurement of HbA_{1C} using the enzymatic assay (Corporation)

3) Cation-exchange high-performance liquid chromatography

Cation-exchange high-performance liquid chromatography is a powerful technique used for the measurement of HbA_{1C} content in clinical settings. CE-HPLC offers a reliable and accurate method for the separation and quantification of HbA_{1C} from other forms of hemoglobin present in a blood sample. In CE-HPLC-based HbA_{1C} measurement, the blood sample is prepared by lysing the red blood cells to release the hemoglobin. The hemoglobin is then injected onto a chromatographic column where it interacts with a stationary phase. A mobile phase, typically a solvent mixture, is pumped through the column under high pressure, allowing for the separation of different hemoglobin components. HbA_{1C}, with its unique chemical structure resulting from glycation, exhibits distinct chromatographic behavior compared to other forms of hemoglobin. This enables its specific identification and quantification. As the hemoglobin components elute from the column, a detector measures the absorbance at a specific wavelength, generating a chromatogram. By comparing the peak corresponding to HbA_{1C} with the total hemoglobin peak or other calibration standards, the percentage or ratio of HbA_{1C} can be determined (**Figure 8**).

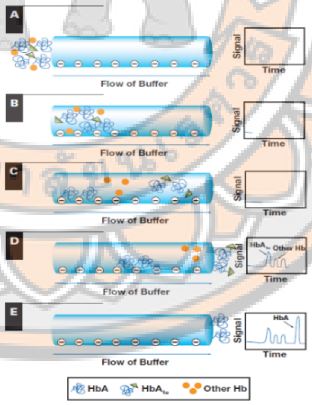


Figure 8 Separation of hemoglobin molecules using CE-HPLC based on different charges (Rhea & Molinaro, 2014)

4) Affinity chromatography

Affinity chromatography is a powerful laboratory technique used to isolate and purify specific proteins or molecules from complex mixtures. It involves the use of a column packed with a resin containing a ligand that selectively binds to the target molecule of interest. The target molecule selectively interacts with the immobilized ligand as the mixture is passed through the column, while other molecules

are washed away. A specific buffer or reagent is then used to elute the bound target molecule from the column. For the purpose of measuring HbA_{1C}, boronated affinity chromatography uses the binding of boron to the special cis diol configuration created by stable glucose attachments to Hb. This method allows for the measurement of all four stable species of HbA_{1C}. These species include glycated and non-glycated forms of HbA_{1C}. The combined measurement of these four species is often referred to as "Total HbA_{1C}" or "True HbA_{1C}." By comparing the glycated portion to the total, the results obtained from affinity chromatography are expressed as a percentage of HbA_{1C} (**Figure 9**). This provides valuable information about the extent of glycation and allows for the accurate assessment of HbA_{1C} levels. Affinity chromatography, particularly boronated affinity chromatography, offers a reliable and specific method for HbA_{1C} measurement, enabling researchers and clinicians to evaluate glycemic control and monitor diabetes management effectively (Gupta et al., 2017).

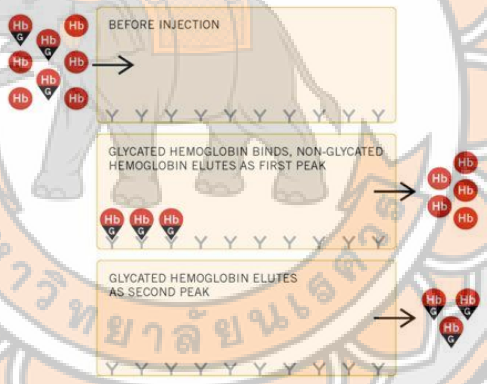


Figure 9 Affinity chromatography for HbA_{1C} measurement (TrinityBiotech, 2023)

10. HbA_{1C} detection interference

HbA_{1C} detection can be affected by various factors that may introduce interference and impact the accuracy of the measurement. Some common sources of interference include:

1) **Hemoglobin variants:** Certain genetic variations can lead to the production of abnormal hemoglobin variants, which may affect the HbA_{1C} measurement. These variants can interfere with the binding of antibodies or alter the glycation process, resulting in inaccurate HbA_{1C} values (**Table 3**).

Table 3 Effects of hemoglobin variants (Rhea & Molinaro, 2014)

Method	Principle	Advantages	Challenges
Enzymatic assay	Uses an enzyme that specifically cleaves the glycated N-terminal valine to measure HbA_{1C}	Hb variants do not interfere with analysis	Unable to detected Hb variants
Capillary electrophoresis	Separates Hb species based on charge and hydrodynamic volume	High chromatographic resolution and the resulting ability to delete many Hb variants	Throughput
Cation-exchange HPLC	Separates Hb species based on a different charge	Capacity to identify the foremost common Hb variations	Prone to interference by Hb variants that coelute with peaks of interest
Turbidimetric immunoassay	Utilizes an antibody directed against the ß-chain's glycated N-terminus (typically the first 4–10 amino acids)	Using newer-generation assays, the most prevalent Hb variants had no impact on the analytical results.	Unable to detect Hb variants; newer-generation antibodies still susceptible to interference from rare Hb variant
Boronate affinity HPLC	Glycohemoglobin binds affinity resin while no glycated Hb passes through the column	Minimal analytical interference from Hb variants	Measure all glycated Hbs, not just HbA_{1C}; unable to detect Hb variants

2) Hemoglobinopathies: Hemoglobinopathies are hereditary disarranges that influence the structure or generation of hemoglobin. Conditions like sickle cell frailty or thalassemia can impact the HbA_{1C} estimation due to changes in hemoglobin composition and glycation designs.

3) Hemolyzed samples: Hemolysis, the breakdown of red blood cells, can release intracellular components, including hemoglobin, into the bloodstream. Hemolyzed samples can introduce excess free hemoglobin, which may interfere with HbA_{1C} measurement by affecting the antibody-antigen interaction or altering the chromatographic separation.

4) Bilirubin and lipemia: High levels of bilirubin (a yellow color determined from the breakdown of ruddy blood cells) or lipids (fats) within the blood can meddle with HbA_{1C} estimation by interferometer with the location strategy or influence the absorbance of light utilized for evaluation.

5) Medications and interfering substances: Certain medications, such as high-dose vitamin C or aspirin, and interfering substances present in the blood, such as high levels of uric acid or acetaminophen, can potentially affect HbA_{1C} measurement and lead to inaccurate results.

11. International organization for standardization of reference material (RMs)

International Organization for Standardization (ISO) 17034 (General requirements for the competence of reference material producers) is an international standard that outlines general requirements for ensuring the competence and consistency of reference material producers. This standard provides guidelines and criteria for developing reference materials (RMs), which are essential tools used for maintaining quality control, calibration, and measurement accuracy across various industries and laboratories. ISO 17034 outlines the requirements for the development, production, and certification of RMs, ensuring that they are fit for their intended purpose. It covers aspects such as the characterization, homogeneity, stability, and traceability of RMs, as well as the competence of the RM producer. Compliance with ISO 17034 ensures that RMs are produced under a systematic and controlled process, following established quality management principles. This standard helps to establish confidence in the accuracy, reliability, and comparability of RMs, facilitating their use

in various measurement applications and supporting reliable and consistent results. By adhering to ISO 17034, reference material producers demonstrate their commitment to producing high-quality RMs that meet the needs of end-users, contribute to measurement accuracy, and enhance the overall quality assurance practices in laboratories and industries (Trapmann et al., 2017).

International Organization for Standardization (ISO) Guide 35:2017 is a standard developed by the International Organization for Standardization (ISO) titled "Reference Materials – Guidance for Characterization and Assessment of Homogeneity and Stability." This guide provides guidelines and recommendations for the characterization, assessment, and documentation of the homogeneity and stability of RMs.

Reference materials (RMs) are essential tools used in various fields, including laboratory testing, quality control, and calibration. The accuracy and reliability of measurements depend on the proper characterization and assessment of the RMs used. ISO Guide 35:2017 aims to ensure that RMs meet the necessary requirements for their intended use.

1) Characterization of RMs: The guide outlines methods and procedures for characterizing RMs, including the determination of their physical, chemical, and biological properties. It emphasizes the importance of using appropriate measurement methods and ensuring traceability to international measurement standards.

2) Homogeneity assessment: Homogeneity refers to the uniformity of an RM, ensuring that it is representative of the target population. ISO Guide 35:2017 provides guidelines for assessing homogeneity, including the design and implementation of sampling plans and statistical evaluation techniques.

3) Stability evaluation: Stability is crucial for RMs to maintain their properties over time. The standard describes methods for evaluating the stability of RMs, including storage conditions, stability testing protocols, and the determination of shelf-life.

4) Uncertainty evaluation: ISO Guide 35:2017 emphasizes the importance of evaluating and reporting measurement uncertainties associated with

RMs. It provides guidance on uncertainty estimation methods and the documentation of uncertainty budgets.

5) Preparation of characterization reports: The standard outlines the essential elements to be included in characterization reports for RMs. This includes detailed information on the methods used, results obtained, uncertainty estimates, and other relevant information.

International Organization for Standardization (ISO) Guide 35:2017 plays a crucial role in ensuring the quality and reliability of RMs used in various fields, such as calibration, quality control, and research. By adhering to the guidelines outlined in the standard, RM producers and users can have confidence in the accuracy and traceability of their measurements, leading to improved consistency and comparability of results (Ellison & Botha, 2018).

12. Commutability

The commutability of HbA_{1C} materials refers to the ability of standard reference material (RM) to produce equivalent results across different laboratories and measurement methods. Commutability is important in ensuring that HbA_{1C} measurements are accurate and consistent across different clinical laboratories and devices, which is essential for the diagnosis, monitoring, and management of diabetes. Several reference materials have been developed to assess the commutability of HbA_{1C} measurements, including the IFCC primary reference material and the National Institute of Standards and Technology (NIST) standard RM. Using commutable HbA_{1C} RMs improves the accuracy and reliability of HbA_{1C} measurements and helps to minimize errors and inter-laboratory variability. It is important for laboratories to participate in external quality assurance programs and use commutable RMs to ensure that their HbA_{1C} measurements are accurate and dependable. This ensures that patients receive consistent, high-quality care regardless of which laboratory or device is used for HbA_{1C} measurement (Mohammadi & Norozi, 2016).

EP14-A4 is a guideline provided by the Clinical and Laboratory Standards Institute (CLSI) titled “Evaluation of Commutability of Processed Samples.” Commutability refers to the property of an RM or external quality assessment (EQA) sample to behave in a similar manner to patient samples when subjected to a particular laboratory test or measurement procedure. The purpose of EP14-A is to provide a

framework for evaluating the commutability of processed samples, which are typically used as reference materials or EQA samples in laboratory testing. Commutability assessment is crucial to ensure the accuracy and reliability of laboratory results, as it helps determine whether the RM or EQA sample can effectively mimic patient samples in a specific testing system. The guideline outlines a systematic approach for evaluating commutability, including the following key steps:

Selection of candidate materials: Suitable RMs or EQA samples that are representative of patient samples and cover the desired range of analyte concentrations should be selected.

Processing of candidate materials: The candidate materials are processed in a manner that replicates the typical pre-analytical steps involved in patient sample handling, such as collection, transport, storage, and preparation. This may include factors like freeze-thaw cycles, stability conditions, and centrifugation protocols.

Comparison of candidate materials: The processed candidate materials are then compared to patient samples using the same measurement procedure or assay. Statistical analyses are performed to assess the agreement between the candidate materials and patient samples, considering factors like bias, linearity, and precision.

Interpretation of results: The results of the commutability assessment are interpreted to determine whether the candidate materials demonstrate acceptable commutability. Acceptance criteria are established based on clinical and analytical considerations. By following the guidelines outlined in EP14-A4, laboratories can evaluate the commutability of processed samples used as RMs or EQA samples. This helps ensure that the materials used in proficiency testing or quality control programs accurately reflect the behavior of patient samples, leading to more reliable and comparable laboratory results.

13. Parameters influence the quality of HbA_{1C} blood materials

The parameters that affect the measurement of HbA_{1C} content in blood materials are presented in **Table 4**.

Table 4 Parameters influence the quality of HbA_{1C} measurement

Parameter	Turbidimetri immunoassay	Enzymatic assay	Cation-exchange HPLC	Boronate affinity HPLC	Capillary electrophoresis
pH	Not available	Not available	Not available	Not available	Not available
Sample hemolysis	Not available	Separation of glucose molecules of each hemoglobin (Reference: Thailand of NEQA HbA_{1C} testing)	Not available	Not available	Not available
Turbidity	Increase in background intensity of light measurement (Hypothesis)	Increase in background intensity of light measurement (Hypothesis)	Not available	Not available	Not available
Debris cells	Not available	Not available	The increasec presence of peaking in chromatogra ms (Reference: Thailand of NEQA HbA_1 testing)	Not available	Not available

1) The measurement of HbA_{1C} can indeed be affected by turbidity. In the context of HbA_{1C} measurement using the immunoassay principle, which employs antibodies specific to the ß-N-terminal of HbA_{1C} bound with latex particles, turbidity arising from the interaction of the antigen-antibody complex is measured using the

turbidimetric inhibition immunoassay technique. Turbidity caused by lipids or blood hemolysis can lead to increased background noise, decreased signal-to-noise ratio, and altered light scattering patterns, all of which can result in distorted or inaccurate HbA_{1C} measurements.

2) pH can potentially affect HbA_{1C} measurements in certain circumstances. The impact of pH on HbA_{1C} can vary depending on the specific method or assay used for HbA_{1C} testing. HbA_{1C} is formed through a process known as glycation, where glucose molecules bind to hemoglobin in red blood cells. The rate of this glycation reaction can be influenced by the pH of the surrounding environment. However, within the typical pH range in which HbA_{1C} measurements are performed (around neutral pH), there is generally no significant effect on the glycation reaction. Additionally, pH also plays a role in the formation of the antigen-antibody complex in immunoassays (Reverberi & Reverberi, 2007). Moreover, pH is involved in the dissociation of molecules in the column for the separation of HbA_{1C} molecules (2015). If the pH is not suitable, it may have an impact on the measurement of HbA_{1C}.

3) Sample hemolysis can potentially affect enzymatic HbA_{1C} measurements. The Enzymatic Assay method uses an enzyme called protease to separate the beta-hemoglobin of HbA_{1C}, initiating a reaction with Fructosyl Peptide Oxidase (FPOD), which produces hydrogen peroxide (H_2O_2) and generates a turbidity measurement proportional to the HbA_{1C} concentration relative to the total hemoglobin (V., 2016). This method allows for the measurement of HbA_{1C} and total hemoglobin in a reduced manner when using fully representative frozen and dried blood samples. To minimize testing inaccuracies, the hemolysate testing mode should be employed.

4) The presence of cell debris can potentially impact HbA_{1C} measurements conducted using the CE-HPLC method. In the CE-HPLC-based analysis of HbA_{1C}, the separation and quantification of glycated hemoglobin from other hemoglobin components relies on the interaction between the sample and the stationary phase in the chromatographic column. Cell debris, such as fragments of red blood cells or other cellular components, can introduce unwanted particles and impurities into the sample. These particles may interact with the stationary phase, leading to clogging or contamination of the column. Additionally, the presence of cell debris in the chromatogram can result in an increased baseline, reducing the proportional

measurement of HbA_{1C}. This can have a negative impact on the accuracy and precision of HbA_{1C} measurements.

The measurement of HbA_{1C} using CE-HPLC (Arkray HA8180 analyzer) shows variations in cell debris among different types of materials (P1-2). The study results are depicted in **Figure 10**, which displays chromatograms.

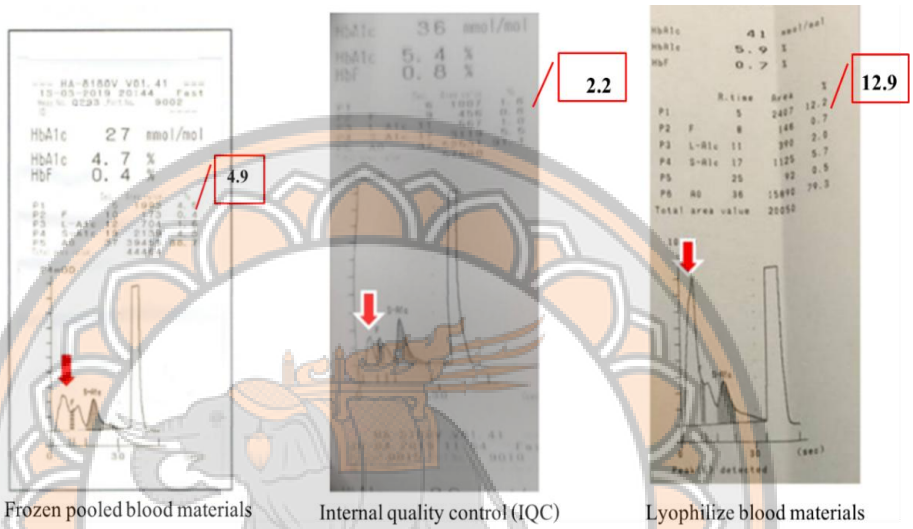


Figure 10 Analyze the number of debris cells in blood using CE- HPLC (Arkray HA8180 analyzer)

14. The selection of measurement procedures for studying commutability

The criteria for selecting a measurement procedure for comparing the results of HbA_{1C} measurements from two methods are as follows:

1) The laboratory should be certified by NGSP.
2) The measurement procedure should provide HbA_{1C} results similar to the IFCC reference measurement procedure.

Evaluation of laboratory proficiency through the EQA EurA_{1C} program by IFCC

The laboratory proficiency assessment is conducted by the International Federation of Clinical Chemistry (IFCC), which organizes the External Quality Assessment (EQA) EurA1c program. EQA EurA1c evaluates the performance of laboratories against the target values determined by the IFCC reference measurement procedure. The differences between the mean of measured values and the target values (Bias) for each measurement procedure are shown in **Table 5**

Table 5 Results per measurement procedure from the EQA EurA1c 2021 (IFCC, 2021)

Measurement procedure	Lyophilized hemolysate materials	Fresh blood materials	Frozen pooled blood materials	Non-DM blood materials	*In vitro* glycation blood materials
Turbidimetric immuno assay (Cobas c 513)	N=17 Bias=**+2.7** CV=4.4	N=34 Bias=+1.6 CV=3.0	Not available	Not available	Not available
Ion exchange HPLC (Tosoh G11)	N=113 Bias=+0.5 CV=3.1	N=116 Bias=+1.6 CV=1.9	Not available	Not available	Not available
Boronate affinity HPLC (Premier Hb9210)	N=23 Bias=-1.2 CV=4.1	N=29 Bias=+1.5 CV=3.8	Not available	Not available	Not available
Enzymatic assay (Alinity c)	N=22 Bias=**-3.8** CV=5.3	N=15 Bias=+0.3 CV=1.2	Not available	Not available	Not available

The evaluation of each measured procedure using lyophilized hemolysate materials and fresh blood materials sets acceptance criteria of biases < 2 mmol/mol and CVs < 6%. The results of the measured procedures show low biases when measuring HbA_{1C} using CE-HPLC and BA-HPLC.

CHAPTER III

RESEARCH METHODOLOGY

This study evaluated six processed blood materials (PBMs), including:

- Four frozen pooled blood samples (FPBs)
- One single donor blood (SBD)
- One *in vitro* glycated blood (IGB)

In addition, an unprocessed native blood samples group comprising four fresh whole blood samples (FWBs) was included in a prospective cohort study.

Hemoglobin A_{1C} content measurement

The HbA_{1C} content of the four commutable FPBs, measured using the EN, was traceable to the IFCC secondary RMP. A total of 24 CBSs and 10 BMs were analyzed for HbA_{1C} content using five measurement procedures:

1. Enzymatic assay
2. Capillary electrophoresis
3. Cation-exchange HPLC
4. Turbidimetric immunoassay
5. Boronate affinity HPLC

Hemoglobin typing

Hemoglobin typing of CBSs and FPBs was performed using CE.

Ethics approval

Written informed consent was obtained from all volunteers, and the study protocol was approved by the Human Research Ethics Committee of Naresuan University, Thailand (COA No. 269/2023; IRB No. P1-0093/2566)

Commutability evaluation

The commutability of PBMs was assessed following the CLSI EP14-A4 guidelines. Non-commutable blood materials were compared with commutable ones to identify factors influencing HbA_{1C} measurements.

Research Structure

Study 1: Investigation of commutability of blood materials

Study 2: Determination of parameters that affect HbA_{1C} measurement

Study 3: Adjust the parameters of the blood materials that affect HbA_{1C} measurement

Study 4: Assess of homogeneity and stability of blood materials

The study was divided into four key areas of investigation

Study 1: Investigation of commutability of HbA_{1C} blood materials

1. Sample preparation

1.1 Clinical blood samples

Twelve milliliters of fresh whole blood in K_3-ethylene diamine tetra acetic acid (EDTA) tubes were collected from 25 volunteers with HbA_{1C} content ranging from 5.0–12.00%. All samples were stored at 2–8 °C until measurement.

1.2 Hemoglobin A_{1C} blood materials

1.2.1 Unprocessed native blood samples

Fresh whole blood materials (FWBs) from individual donors were classified into four categories based on their HbA_{1C} content:

- FWB1: HbA_{1C} content range of 5.0–5.9%
- FWB2: HbA_{1C} content range of 6.0–7.9%
- FWB3: HbA_{1C} content range of 8.0–9.9%
- FWB4: HbA_{1C} content range of 10.0–12.0%

All samples were stored at 2–8°C until analysis to ensure stability and preserve HbA_{1C} content.

1.2.2 Processed blood materials (PBMs)

Processed blood materials were prepared as follows:

1) Frozen pooled blood materials (FPBs)

Frozen pooled blood materials (FPBs) were generated from remnant EDTA blood and selected based on HbA_{1C} contents. The samples were categorized into four groups:

- FPB1: HbA_{1C} content range of 5.0–5.7%
- FPB2: HbA_{1C} content range of 6.0–7.0%
- FPB3: HbA_{1C} content range of 8.0–9.0%
- FPB4: HbA_{1C} content range of 10.0–12.0%

Samples within each category were pooled, aliquoted into 0.5 ml tubes, and stored at -70°C for up to one year to maintain integrity (Suparak et al., 2022).

2) Single-donor blood material (SDB)

Single-donor blood material (SDB) was prepared from individual donors with HbA_{1C} content ranging from 5.0–7.0%. The blood samples were centrifuged to separate plasma, washed with 0.85% saline, and adjusted to a

hematocrit (HCT) of 40% using citrate phosphate dextrose adenine (CPDA-1) solution (Wongsri, 2018). These samples were stored at 2–8°C to ensure stability for further testing.

3) *In vitro* glycated blood material (IGB)

In vitro glycated blood material (IGB) was prepared by incubating donor erythrocytes with D-glucose in phosphate-buffered saline (PBS) containing sodium azide at 37°C (W Duanginta et al., 2023). The glycated samples were then aliquoted into 0.3 ml tubes and stored at 2–8°C for future use (W Duanginta et al., 2023; Wongsri, 2018).

2. HbA_{1C} measurement

Twenty-five CBSs and ten HbA_{1C} blood materials were stored at 2–8°C. The HbA_{1C} content was measured within five days of blood specimen collection using five measurement procedures. The HbA_{1C} measurements were conducted by laboratories certified by NGSP (IFCC, 2021) or accredited with ISO 15189.

The laboratories selected for this study were either NGSP-certified or employed NGSP-certified procedures. The devices used for HbA_{1C} measurement, along with their measurement procedure ologies and testing laboratories, are listed in **Table 6**.

Table 6. Analytical tools and equipment for HbA_{1C} measurement

Instrument	Measurement procedure	Manufacturer	Tested by
Cobas c513 (Roche Diagnostics, Rotkreuz Switzerland)*	Turbidimetric immunoassay	Roche Diagnostics GmbH	Immunology Laboratory, Ramathibodihospital-Mahidol University, Bangkok, Thailand**
Tosoh G11 (Tosoh Bioscience, Inc., Tokyo, Japan)*	Cation-exchange HPLC	Tosoh Corporation	Siriraj Hospital - Mahidol University, Bangkok, Thailand**
Premier Hb 9210 (Trinity Biotech, Wicklow, Ireland)*	Boronate affinity chromatography	Trinity Biotech	Immunology Laboratory, Ramathibodihospital-Mahidol University, Bangkok, Thailand

Instrument	Measurement procedure	Manufacturer	Tested by
Alinity c analyzer (Abbott Laboratories, Lake County, IL, USA)*	Enzymatic assay	Abbott Laboratories	Immunology Laboratory, Ramathibodihospital-Mahidol University, Bangkok, Thailand**
Sebia CAPILLARYS 2 (Sebia, Lisses, France)*	Capillary electrophoresis	Sebia	Buddhasothorn Hospital, Chachoengsao, Thailand

* NGSP certification procedure (NGSP, 2023)

** List of NGSP-certified laboratories

The HbA_{1C} content of each sample was measured using five different measurement procedures performed at specialized laboratories in Thailand. These procedures included the enzymatic assay (EN) on the Alinity c analyzer (Abbott Laboratories, Lake County, IL, USA), the turbidimetric immunoassay (TI) on the Cobas c513 (Roche Diagnostics, Rotkreuz, Switzerland), and boronate affinity chromatography (BA-HPLC) on the Premier Hb9210 (Trinity Biotech, Wicklow, Ireland), all conducted at the Immunology Laboratory, Ramathibodi Hospital-Mahidol University, Bangkok. Additionally, cation-exchange high-performance liquid chromatography (CE-HPLC) was performed using the Tosoh G11 (Tosoh Bioscience, Inc., Tokyo, Japan) at Siriraj Hospital-Mahidol University, Bangkok, while capillary electrophoresis (CE) was conducted using the Sebia CAPILLARYS 2 (Sebia, Lisses, France) at Buddhasothorn Hospital, Chachoengsao. Each sample was analyzed in triplicate test using the same reagent lot across all measurement procedures to ensure consistency and accuracy in the results.

Selection of reference standard procedures for commutability study

1) Comparison of HbA_{1C} measurements from various procedures of FPB to select a reference measurement procedure

Prior to conducting this study, four FPBs were prepared. These samples were provided by the Department of Medical Sciences, Ministry of Public Health, Thailand, as part of their proficiency testing program. The HbA_{1C} content of these FPBs was assigned using the IFCC secondary reference measurement procedures (IFCC Secondary RMP) from the IFCC organization in the Netherlands. The target values from four FPBs were compared with HbA_{1C} measurements obtained using five measurement procedures to identify a suitable representative reference standard procedure. Bias for each procedure was calculated using the formula:

$$\text{Bias} = \text{xi} - \text{Target value}$$

where xi represents the mean HbA_{1C} result from each procedure. According to the CAP guidelines (Updated 5/24), a bias within the range of ±6% was considered acceptable (CAP, 2024). The selected procedure served as a representative of the secondary reference procedures established by the IFCC in the Netherlands.

Table 7. Comparison of HbA_{1C} measurements from various procedures of FPB to select a representative reference measurement procedure

HbA_{1C} blood material	IFCC Target value	Turbidimetric immunoassay		Enzymatic assay		Boronate affinity HPLC		Capillary electrophoresis		Cation-exchange HPLC	
		Mean	Bias	Mean	Bias	Mean	Bias	Mean	Bias	Mean	Bias
FPB1	5.21	5.40	0.19	5.27	0.06	5.04	-0.17	5.20	-0.01	5.30	0.09
FPB2	6.62	6.75	0.13	6.58	-0.04	6.23	-0.39	6.57	-0.05	6.77	0.15
FPB3	8.26	8.56	0.30	8.22	-0.04	7.85	-0.41	8.37	0.11	8.43	0.17
FPB4	11.03	11.48	0.45	11.00	-0.03	10.64	-0.39	11.30	0.27	11.13	0.10

Abbreviations: FPB; frozen pooled blood materials

The mean bias from triplicate tests of FPBs, when compared to the target values from the IFCC procedure in the Netherlands, fell within acceptable limits, as shown in **Table 7**, according to CAP guidelines (mean bias within ±6%) (CAP, 2024). However, the BA-HPLC procedure exhibited a high bias that exceeded acceptable

limits. Conversely, the enzymatic assay demonstrated values closely aligned with the IFCC measurement procedure and was thus selected as the reference measurement procedures for commutability assessment.

2) Traceability of HbA_{1C} measurements

The evaluation of procedure performance was carried out using statistical comparison principles to assess the alignment of measured values with certified reference values. The analysis focused on the absolute difference (Δ_m) and the combined uncertainty (u_Δ), following quality assurance and validation guidelines such as ISO 15189 and ISO Guide 35.

The absolute difference (Δ_m) between the mean measured value (c_m) and the certified reference value (c_{CRM}) was calculated using the formula:

$$\Delta_m = |c_m - c_{CRM}|$$

When:

- c_m: The mean value obtained from three test results conducted using the EN procedure at the Immunology Laboratory, Ramathibodi Hospital-Mahidol University, Bangkok, Thailand.
- c_{CRM} : The target value provided by the IFCC secondary RMP.

To account for uncertainties in both the measurement result and the certified reference value, the combined uncertainty (u_Δ) was calculated using the formula:

$$u_\Delta = \sqrt{u_m^2 + u_{CRM}^2}$$

When:

- u_m: Uncertainty associated with Immunology Laboratory, Ramathibodi Hospital-Mahidol University, Bangkok, Thailand
- c_{CRM} : The target value provided by the IFCC secondary RMP.

The results were interpreted by comparing Δ_m with the expanded uncertainty (u_c) of the certified value.

- If $\Delta_m \leq U_c$, the measurement procedure was deemed consistent with the certified value, indicating no significant difference.
- If $\Delta_m > U_c$, the procedure's performance was found inconsistent, suggesting potential systematic errors.

This evaluation, leveraging both the EN procedure and IFCC standards, ensured a rigorous assessment of the measurement procedure's reliability and alignment with certified reference materials. This approach provides a robust framework for quality assurance, ensuring metrological traceability and adherence to international standards.

3. Hemoglobin typing

A total of twenty-five CBSs and ten HbA_{1C} blood materials were analyzed for hemoglobin typing. Testing was performed using capillary electrophoresis (CE) on the Sebia CAPILLARYS 2 (Sebia, Lisses, France) at Bangkok Hospital Phitsanulok Company Limited. The results were provided as chromatograms and reported in terms of hemoglobin typing, ensuring accuracy and comprehensive assessment of blood sample characteristics.

4. Data analysis

To assess commutability, pairwise comparisons were performed between the five measurement procedures. The analysis included the following steps:

1. Deming regression: Regression lines were calculated between paired measurement procedures, accounting for errors in both variables, to evaluate the relationship between results.

2. 95% Prediction Interval (PI): Prediction intervals were generated for each pair of procedures using 25 CBSs based on the CLSI EP14-A4 formula. These intervals represented the expected range of variability for CBS results. based on the CLSI EP14-A4 formula.

3. Plotting results: The HbA_{1C} results for the ten PBMs were plotted against the PIs for each pair of procedures to assess commutability.

Commutability assessment:

- PBMs with results falling within the 95% PI were classified as commutable PBMs.
- PBMs with results outside the 95% PI were identified as non-commutable PBMs.

This approach provided a robust framework for evaluating the consistency and reliability of HbA_{1C} measurement across different procedures.

Study 2: Determination of parameters that affect HbA_{1C} measurement

1. Measurement of parameters

Six commutable PBMs (FWB1, FWB2, FWB3, FWB4, FPB1, and SBD) and four non-commutable PBMs (FPB2, FPB3, FPB4, and IGB) were tested for hematocrit, hemolysis, pH, and debris cell content. Hematocrit measurements were performed using an iFuge HCT centrifuge (Neuation), with samples centrifuged at 400 rcf for 20 minutes to separate the red blood cells (RBCs). Post-centrifugation, the supernatant was used for optical density measurements with an SP-8001 UV/visible spectrophotometer (Metertech) and pH measurements using the Humming Probe (Ultram). All measurements were conducted in triplicate to ensure precision and reliability.

Hemolysis index calculation

The hemolysis index was calculated as the percentage of hemolysis in the test sample relative to the control group. This was determined by dividing the sample's absorbance at 540 nm by the control's absorbance at 540 nm and multiplying the result by 100. The formula used is:

$$\text{Hemolysis index (\%)} = \frac{\text{Absorbance of sample at 540 nm}}{\text{Absorbance of control (hemolysis) at 540 nm}} \times 100$$

Where:

- Absorbance of sample: The optical density of the test sample (commutable PBMs and non-commutable PBMs) measured at 540 nm.
- Absorbance of control: The optical density of the control sample measured at 540 nm. The control sample was prepared from blood that had undergone freezing and exhibited hemolysis.

Debris cell count calculation

The debris cell count was measured as the percentage of peak FA, A_1A, and A_1B areas in the chromatograms generated by the Tosoh G11 analyzer (Tosoh Bioscience, Inc., Tokyo, Japan). These measurements reflect the presence of specific components within the samples, providing insights into their quality. The formula used is:

$$\text{Debris cells count (\%)} = \frac{\text{Peak area of FA + A1A + A1B}}{\text{Total chromatogram area}} \times 100$$

Where:

- Peak area of FA, A1A, and A1B: The sum of the areas under the peaks representing FA, A1A, and A1B in the chromatogram.
- Total chromatogram area: The total area under all peaks in the chromatogram.

A schematic representation of the procedures used to measure hematocrit, hemolysis, pH, and debris cell count is shown in **Figure 11**.

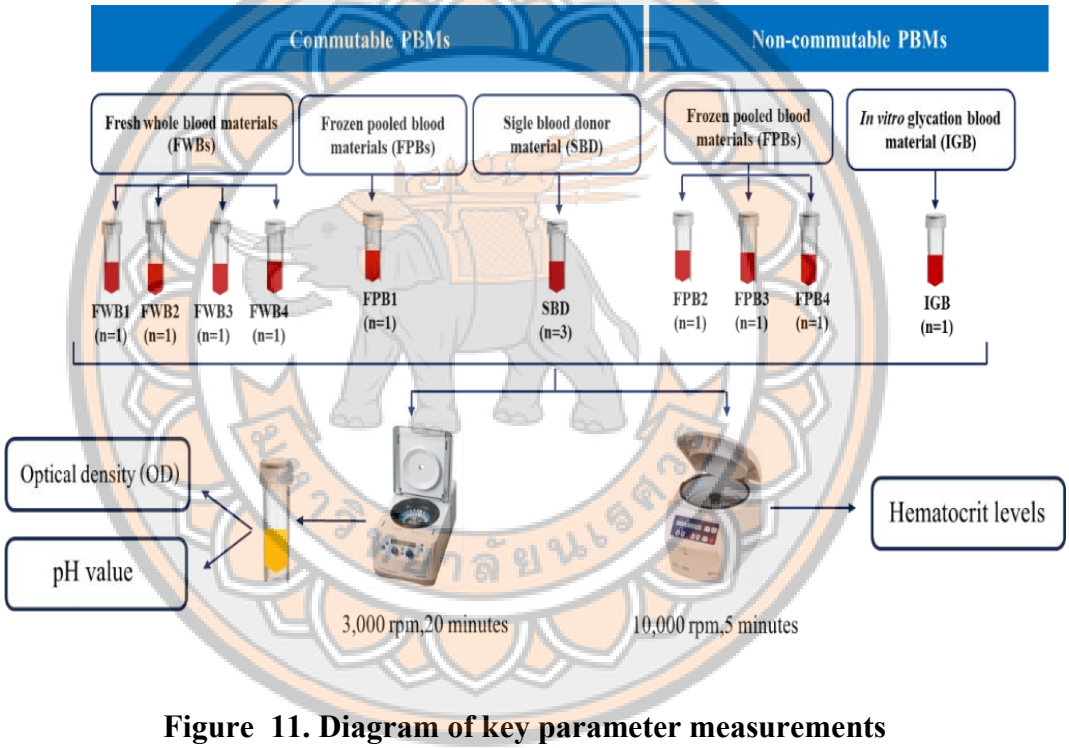


Figure 11. Diagram of key parameter measurements

2. Data analysis

The data analysis for this study involved a systematic approach to evaluate the differences between non-commutable PBMs (FPB2, FPB3, FPB4, IGB) and unprocessed native blood samples (FWB1, FWB2, FWB3, FWB4) based on key parameters. First, the Shapiro-Wilk test was conducted to assess the normality of the data. If the *p-value* was $p > 0.05$, the data were deemed normally distributed, while $p \leq 0.05$ indicated a non-normal distribution. Depending on the data distribution, statistical comparisons were performed using either a paired t-test for normally distributed data or the Mann–Whitney U test for non-normally distributed data.

Additionally, the Kruskal-Wallis test was employed to compare each parameter in PBMs, and the intraclass correlation coefficient was used to determine the correlation between parameters within each PBM.

The relationship between DC and HbA_{1C} content in five production lots of non-commutable FPBs was compared to commutable FPBs using the paired t-test, providing insights into the impact of DC on the commutability of HbA_{1C} measurements.

A significance level of $p < 0.05$ was set as the threshold for identifying statistically significant differences between the two groups. The analysis focused on HI, pH levels, HCT, and debris cell count to pinpoint significant changes specific to non-commutable PBMs. By comparing these parameters between each production lots of non-commutable and unprocessed native blood samples groups, the study aimed to uncover key factors contributing to non-commutability. This approach provided a robust framework for identifying critical differences that impact the usability of PBMs in diagnostic applications.

Study 3: Adjust the parameters of the blood materials that affect HbA_{1C} measurement

The study aimed to modify HbA_{1C} blood materials to improve their compatibility with HbA_{1C} measurement procedure. The criteria for selecting HbA_{1C} blood materials for parameter adjustment were as follows:

1. Non-commutable HbA_{1C} blood materials identified in each measurement procedure were modified to address parameters that impacted HbA_{1C} measurement.

2. Hemoglobin A_{1C} blood materials were prepared in larger quantities to allow for subsequent testing of their homogeneity and stability.

The non-commutable blood materials from each measurement procedure were adjusted in terms of their biological components, such as HI, and pH, and DC which significantly influenced HbA_{1C} measurement. These modified blood materials were then evaluated for commutability across different analytical procedures. Successful modification enabled these materials to be used universally across all HbA_{1C} measurement techniques, ensuring their suitability for laboratory use and enhancing interlaboratory comparability

Study 4: Assess of homogeneity and stability of HbA_{1C} blood materials

Homogeneity test

The homogeneity of the four FPB was tested after preparation. Random samples were collected from the batch, with the number of units determined according to ISO Guide 35 recommendations. The number of units ranged between:

$$\sqrt[3]{N_{prod}} \text{ and } 3 \times \sqrt[3]{N_{prod}}$$

The minimum number of units N_{min} was calculated using the following formula:

$$N_{min} = \max(10, \sqrt[3]{N_{prod}})$$

The experimental assessment included evaluating within-unit heterogeneity and ensuring that the minimum sample size requirement was met. A minimum of $n \geq 10$ units was randomly selected from the final packaging.

Data Analysis

The homogeneity of the PBMs was assessed according to ISO Guide 35 using one-way ANOVA (95% critical values). The results were interpreted as follows:

- If $F_{test} < F_{critical}$, the material is considered homogeneous (no significant difference between units).
- If $F_{test} > F_{critical}$, it indicates heterogeneity (significant difference between units).

This methodology ensured that the PBMs met the required standards for homogeneity and stability, confirming their suitability for use in laboratory settings and distribution. Subsequently, the blood materials were further evaluated for commutability in the next phase of the study.

Stability test

The processed blood materials (PBMs), including four FPBs, were stored at -70 °C for 0, 3, 6, 9 and 12 months. After the storage period, the HbA_{1C} content of the four FPBs was analyzed using the enzymatic assay (EN) on the Alinity c analyzer (Abbott Laboratories, Lake County, IL, USA).

Data Analysis

Stability was assessed through linear regression analysis, where a slope of 0 indicated stability. A paired t-test was used to evaluate whether the slope significantly differed from 0, suggesting potential instability.

This methodology ensured that the PBMs complied with the necessary standards for homogeneity and stability, validating their suitability for laboratory use and distribution. The blood materials were subsequently subjected to commutability evaluation in the next phase of the study.

CHAPTER IV

RESULTS AND DISCUSSION

Study 1: Investigation of commutability of HbA_{1C} blood materials

1. The Hb typing and HbA_{1C} content of 23 CBSs

The study collected twelve milliliters of CBSs from 25 volunteers in K_3-EDTA tubes. The HbA_{1C} content was analyzed using five different procedures: EN (RMP), CE-HPLC, CE, TI, and BA-HPLC, yielding HbA_{1C} content ranging from 4.87% to 11.60% (**Table 8**). The chromatograms for Hb typing in CBSs, analyzed using CE, are shown in **Figure 12**. The HbA_{1C} content for each sample, categorized by Hb typing groups, is illustrated in **Figures 13-15**.

Hemoglobin typing revealed 20 cases of Hb A_2A, 4 cases of Hb E trait, and 1 case of homozygous Hb E. For samples with Hb A_2A typing, HbA_{1C} content ranged from 4.87-11.60% (**Table 8**), whereas samples with Hb EA typing (n = 4) display HbA_{1C} content ranging from 5.11- 9.70% (**Table 9**).

The distribution of data for each Hb typing group was assessed using the Shapiro-Wilk test ($n \leq 50$), which confirmed a normal distribution ($p > 0.05$) for all procedures. Subsequently, differences in HbA_{1C} measurements among the five procedures were analyzed for each Hb typing group. No significant differences were detected among the results obtained from the five procedures, with $p = 0.991$ for Hb A_2A and $p = 0.993$ for Hb EA (**Tables 8–9**).

Table 8. Hemoglobin A_{1C} content of 19 cases of Hb typing A_2A

CBSs	Average ± SD of HbA_{1C} content (%NGSP) (n=3)				
	EN	CE	CE-HPLC	TI	BA-HPLC
CS1	4.92 ± 0.00	4.90 ± 0.00	4.87 ± 0.06	5.08 ± 0.04	4.93 ± 0.14
CS2	5.09 ± 0.02	5.17 ± 0.12	5.20 ± 0.00	5.23 ± 0.00	5.05 ± 0.03
CS3	5.20 ± 0.02	5.37 ± 0.06	5.20 ± 0.00	5.39 ± 0.03	5.29 ± 0.11
CS4	5.68 ± 0.02	5.97 ± 0.06	5.87 ± 0.06	5.86 ± 0.03	5.81 ± 0.03
CS5	6.71 ± 0.08	6.93 ± 0.06	6.70 ± 0.00	6.91 ± 0.01	6.98 ± 0.13
CS6	6.75 ± 0.02	7.07 ± 0.06	6.90 ± 0.00	7.00 ± 0.03	6.97 ± 0.11
CS7	6.80 ± 0.01	7.10 ± 0.10	7.00 ± 0.00	6.99 ± 0.03	6.84 ± 0.08
CS8	6.84 ± 0.05	6.93 ± 0.06	6.87 ± 0.06	7.03 ± 0.03	6.96 ± 0.06
CS9	7.01 ± 0.01	7.27 ± 0.06	7.23 ± 0.06	7.26 ± 0.03	7.33 ± 0.09
CS10	7.15 ± 0.01	7.47 ± 0.06	7.33 ± 0.06	7.44 ± 0.04	7.19 ± 0.10
CS11	7.32 ± 0.02	7.70 ± 0.10	7.60 ± 0.00	7.58 ± 0.02	7.45 ± 0.05
CS12	7.40 ± 0.01	7.57 ± 0.12	7.50 ± 0.00	7.64 ± 0.02	7.64 ± 0.09
CS13	7.46 ± 0.03	7.63 ± 0.15	7.83 ± 0.06	7.75 ± 0.03	7.73 ± 0.05
CS14	8.07 ± 0.06	8.27 ± 0.12	8.27 ± 0.06	8.18 ± 0.08	8.23 ± 0.18
CS15	9.47 ± 0.02	9.87 ± 0.15	9.70 ± 0.00	9.91 ± 0.02	9.95 ± 0.06
CS16	9.79 ± 0.02	10.03 ± 0.12	10.07 ± 0.06	10.26 ± 0.06	10.18 ± 0.12
CS17	10.73 ± 0.02	10.77 ± 0.21	10.97 ± 0.06	11.16 ± 0.01	11.24 ± 0.17
CS18	10.75 ± 0.01	10.97 ± 0.06	11.00 ± 0.10	11.10 ± 0.07	11.12 ± 0.28
CS19	11.24 ± 0.02	11.50 ± 0.20	11.43 ± 0.06	11.60 ± 0.03	11.43 ± 0.19
*P**	0.076	0.130	0.122	0.059	0.044
*p***			0.991		

*Analysis of the normal distribution of HbA_{1C} content in each measurement procedure using the Shapiro-Wilk test.

**Analysis of differences in HbA_{1C} content across five measurement procedures using One-Way ANOVA.

Table 9. Hemoglobin A_{1C} content of 4 cases of Hb E traits

CBSs	Average ± SD of HbA_{1C} content (%NGSP) (n=3)				
	EN	CE	CE-HPLC	TI	BA-HPLC
CS20	5.11 ± 0.01	5.03 ± 0.06	5.40 ± 0.00	5.22 ± 0.09	5.17 ± 0.05
CS21	5.42 ± 0.01	5.30 ± 0.00	5.87 ± 0.06	5.45 ± 0.09	5.45 ± 0.10
CS22	6.50 ± 0.05	6.63 ± 0.15	6.70 ± 0.00	6.69 ± 0.04	6.82 ± 0.04
CS23	8.83 ± 0.02	9.00 ± 0.30	9.70 ± 0.10	8.90 ± 0.04	9.53 ± 0.12
*P**	0.330	0.346	0.235	0.363	0.314
*p***			0.993		

*Analysis of the normal distribution of HbA_{1C} content in each measurement procedure using the Shapiro-Wilk test.

**Analysis of differences in HbA_{1C} content across five measurement procedures using One-Way ANOVA.

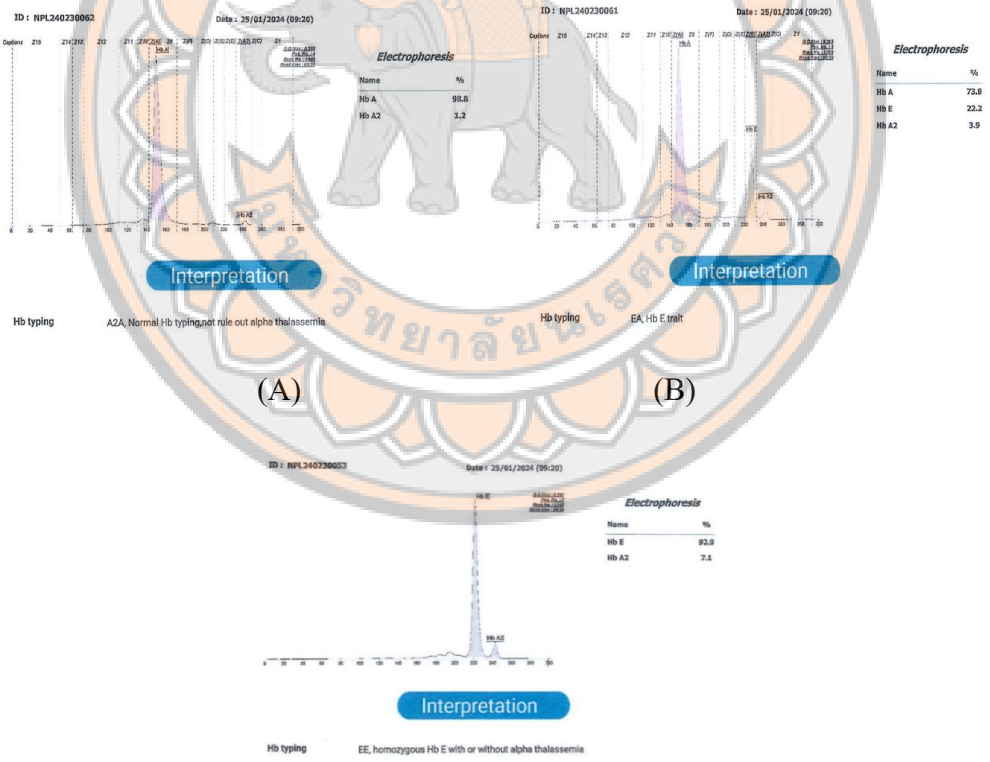


Figure 12. Hemoglobin typing of CBSs (A) normal hemoglobin pattern (B) Hb E trait, and (C) homozygous Hb E with or without alpha thalassemia

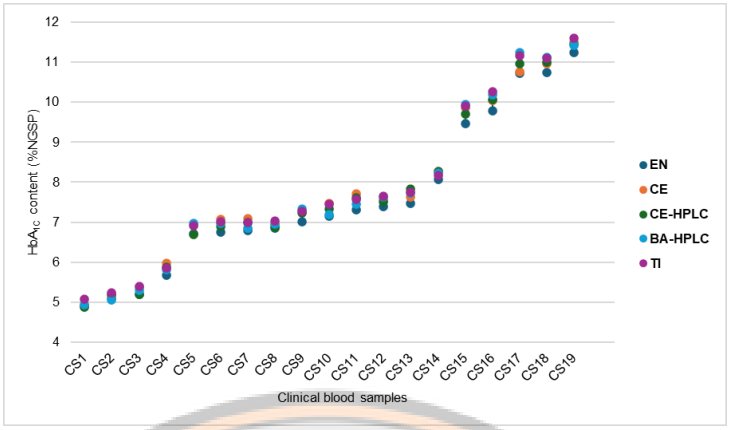


Figure 13. Displays a graph of the HbA_{1C} content measurements in 20 CBSs with

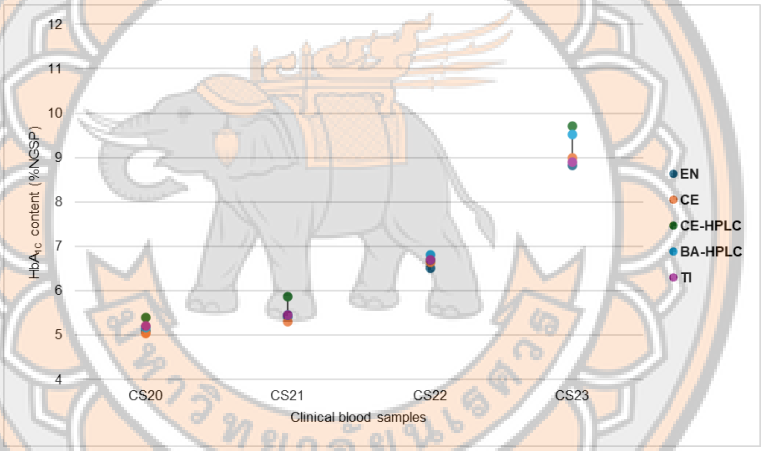


Figure 14. Displays a graph of the HbA_{1C} content measurements in 4 CBSs with Hb typing classified as E trait

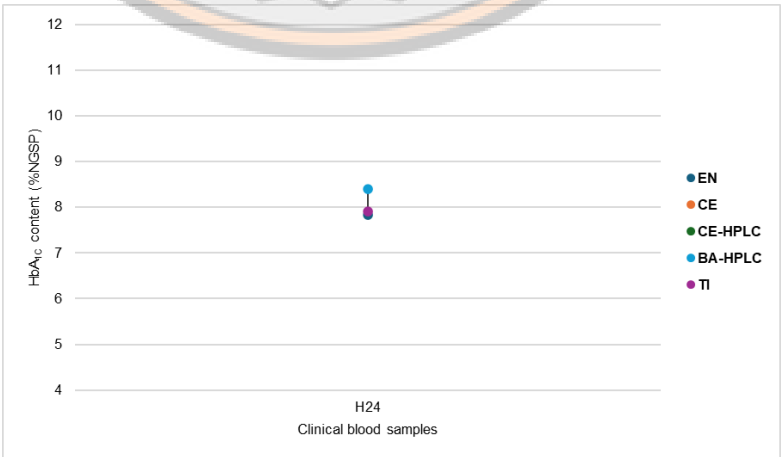


Figure 15. Displays a graph of the HbA_{1C} content measurements in 4 CBSs with Hb typing classified as homozygous Hb E

2. Hemoglobin typing and HbA_{1C} content of 10 HbA_{1C} blood materials

The PBMs, comprising four FPBs, one SBD, and one IGB, and unprocessed native blood samples, were analyzed for Hb typing and HbA_{1C} content. Hb typing could not be determined for the FPBs, while the SBD was identified as Hb A_2A. The HbA_{1C} content of all samples was measured using five different procedures. The detailed results for HbA_{1C} measurements are presented in **Table 10**. The chromatogram of the Hb typing analysis is illustrated in **Figure 16**.

Table 10. Hemoglobin typing and HbA_{1C} content of 10 blood materials

HbA_{1C} blood materials	Hb typing	Average ± SD of HbA_{1C} content (%NGSP)				
		EN	CE	CE-HPLC	TI	BA-HPLC
Processed blood materials						
FPB1	Under terred mined	5.27 ± 0.02	5.20 ± 0.10	5.30 ± 0.10	5.40 ± 0.02	5.04 ± 0.02
FPB2		6.58 ± 0.01	6.57 ± 0.15	6.77 ± 0.06	6.75 ± 0.02	6.23 ± 0.04
FPB3		8.22 ± 0.02	8.37 ± 0.06	8.43 ± 0.06	8.56 ± 0.04	7.85 ± 0.07
FPB4		11.00 ± 0.02	11.30 ± 0.17	11.13 ± 0.12	11.48 ± 0.09	10.64 ± 0.19
SBD	A_2A	5.16 ± 0.02	5.13 ± 0.06	5.03 ± 0.06	5.29 ± 0.02	5.03 ± 0.08
IGB		8.20 ± 0.03	8.00 ± 0.10	7.70 ± 0.00	8.31 ± 0.04	11.30 ± 0.13
Unprocessed blood materials						
FWB1	A_2A	5.09 ± 0.02	5.17 ± 0.12	5.20 ± 0.00	5.23 ± 0.00	5.05 ± 0.03
FWB2		6.84 ± 0.05	6.93 ± 0.06	6.87 ± 0.06	7.03 ± 0.03	6.96 ± 0.06
FWB3		9.47 ± 0.02	9.87 ± 0.15	9.70 ± 0.00	9.91 ± 0.02	9.95 ± 0.06
FWB4		10.75 ± 0.01	10.97 ± 0.06	11.00 ± 0.10	11.10 ± 0.07	11.12 ± 0.28

Abbreviations: A_2A: normal Hb typing; FPB: frozen pooled blood materials; SBD: single blood donor materials; IGB: *in vitro* glycation blood materials; FWB: fresh whole blood materials

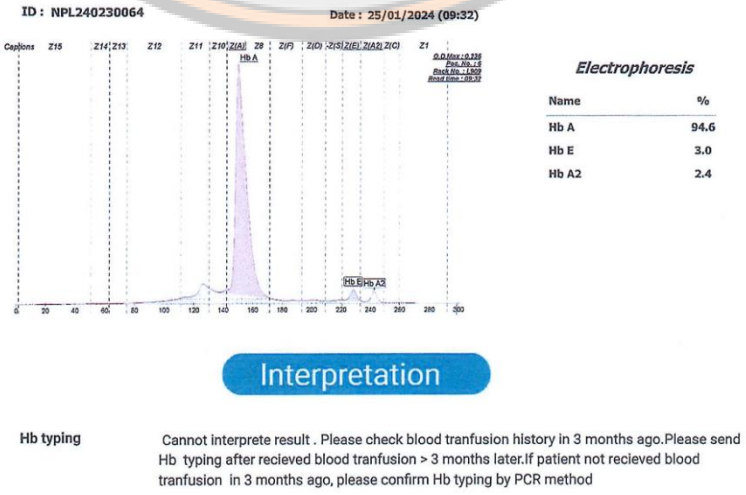


Figure 16. Chromatogram of the hemoglobin typing in FPB

Traceability of HbA_{1C} measurements

The HbA_{1C} content in FPBs was compared using the IFCC secondary RMP and EN procedure performed at Ramathibodi Hospital. As shown in Table 1, the analysis revealed consistent results between the two measurement procedures. The absolute difference (Δ_m) of HbA_{1C} content between the EN procedure and the IFCC secondary RMP fell under the acceptable combined uncertainty of the two procedures ($\Delta_m \leq U_c$), confirming alignment with the IFCC secondary RMP (**Table 11**). These findings confirm the traceability of the EN procedure to the IFCC standard, validating its reliability for HbA_{1C} testing. These results establish the traceability of the EN procedure to the IFCC standard, validating its reliability for HbA_{1C} testing. Consequently, the EN procedure was selected as the reference procedure for the commutability study, ensuring compliance with international standards and methodological precision.

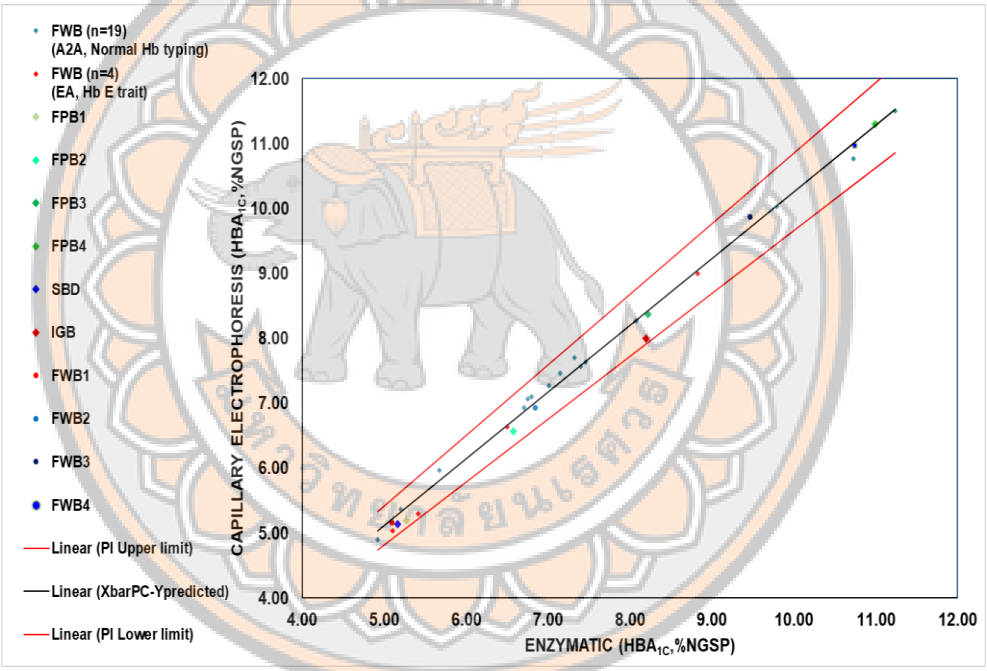
Table 11. Target values of HbA_{1C} content in %NGSP in four FPBs and traceability of the enzymatic assay to the IFCC secondary RMP

HbA_{1C} blood material	**HbA_{1C}**		**Δ_m (%)**	**Interpretation with U_c =2.43 (%)**
	Target value mean ± uncertainty	Enzymatic assay mean ± uncertainty		
FPB1	5.21 ± 0.10 (33.4 ± 1.1)	5.27 ± 2.43 (34.3 ± 3.05)	0.06	Acceptable
FPB2	6.62 ± 0.09 (48.8 ± 1.0)	6.58±2.43 (48.3 ±3.05)	0.04	Acceptable
FPB3	8.26 ± 0.10 (66.8 ± 1.1)	8.22 ± 2.43 (66.0 ± 3.05)	0.04	Acceptable
FPB4	11.03 ± 0.16 (97.1 ± 1.8)	11.00 ± 2.43 (96.7 ± 3.05)	0.03	Acceptable

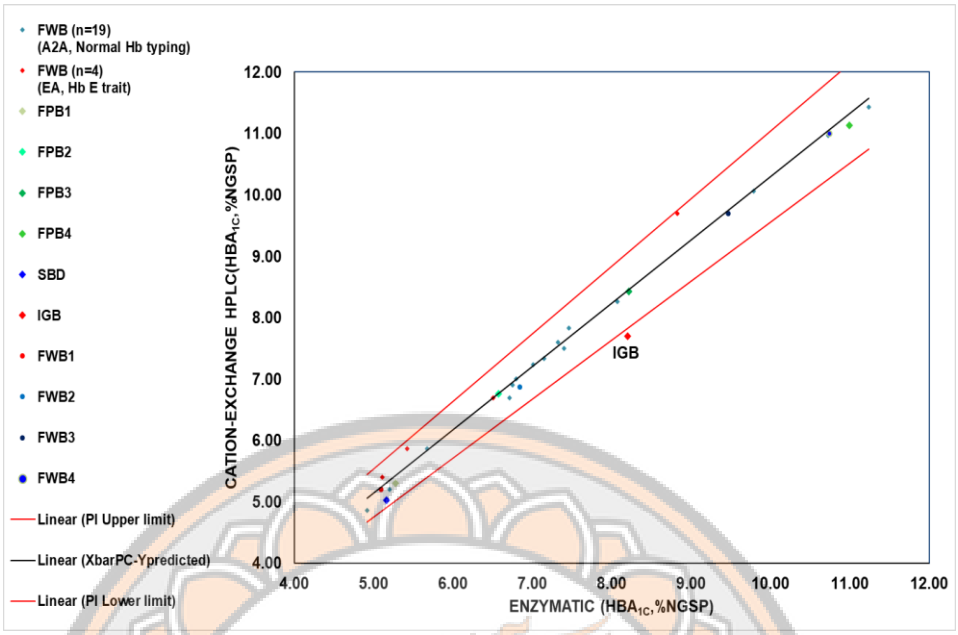
Indicates HbA_{1C} content expressed in mmol/mol, calculated using the formula: HbA_{1C} (mmol/mol) = (10.93*NGSP) - 23.50. U_c is combined uncertainty form for formula: $u_c = \sqrt{u_m^2 + u_{CRM}^2}$. Abbreviations: FPB: frozen pooled blood materials

3. Commutability analysis of HbA_{1C} blood materials

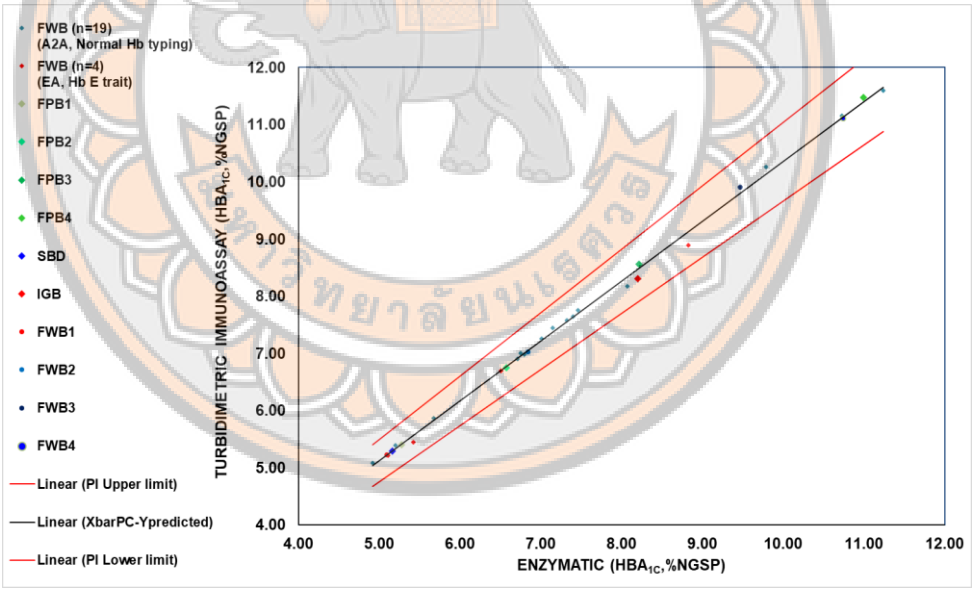
The commutability of FWB and PBMs was assessed using the CLSI approach, with results depicted in **Figure 17** and summarized in **Table 12**. Fresh whole blood materials (FWB1–4), FPB1, and SBD demonstrated commutability across all paired procedures. Frozen pooled blood materials (FPB2 and FPB3) were commutable for EN/TI, EN/CE, and EN/CE-HPLC but not for the EN/BA-HPLC procedure. Among the FPBs, three FPBs demonstrated non-commutability out of the 95% PI when measured HbA_{1C} content compared to BA-HPLC procedure. In contrast, IGB was identified as non-commutable across two paired measurement procedures.



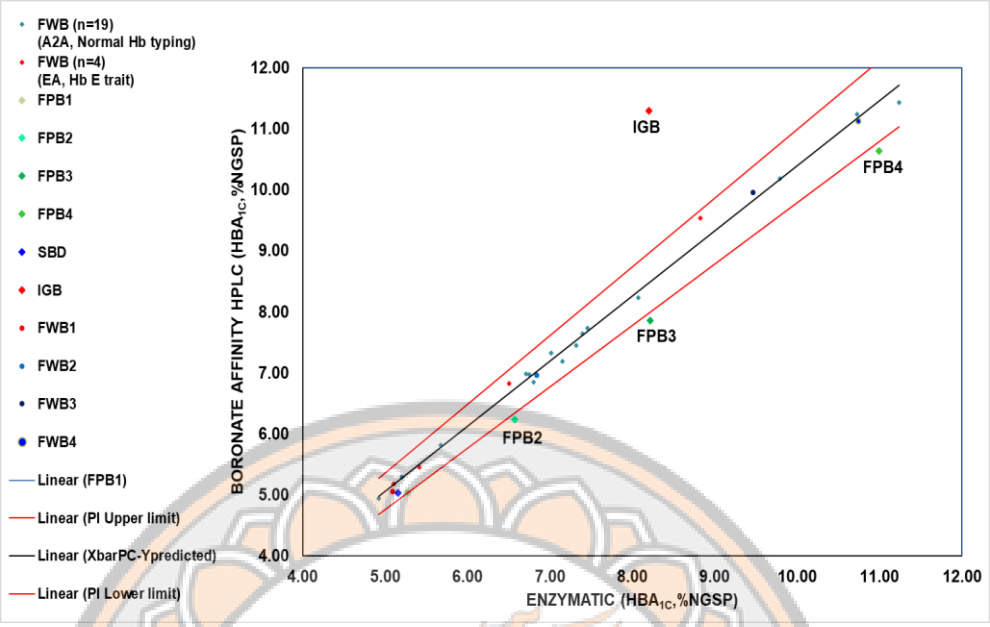
(A)


FWB (n=19)
(A2A, Normal Hb typing)
FWB (n=4)
(EA, Hb E trait)
FPB1
FPB2
FPB3
FPB4
SBD
IGB
FWB1
FWB2
FWB3
FWB4
Linear (PI Upper limit)
Linear (XbarPC-Ypredicted)
Linear (PI Lower limit)
CATION-EXCHANGE HPLC(HBA1C,%NGSP)
ENZYMATIC (HBA1C,%NGSP)
IGB
4.00
5.00
6.00
7.00
8.00
9.00
10.00
11.00
12.00

(B)


FWB (n=19)
(A2A, Normal Hb typing)
FWB (n=4)
(EA, Hb E trait)
FPB1
FPB2
FPB3
FPB4
SBD
IGB
FWB1
FWB2
FWB3
FWB4
Linear (PI Upper limit)
Linear (XbarPC-Ypredicted)
Linear (PI Lower limit)
TURBIDIMETRIC IMMUNOASSAY (HBA1C,%NGSP)
ENZYMATIC (HBA1C,%NGSP)
4.00
5.00
6.00
7.00
8.00
9.00
10.00
11.00
12.00

(C)



(D)

Figure 17. Deming regression plots comparing enzymatic assay with the (A) capillary electrophoresis, (B) cation-exchange HPLC, (C) turbidimetric immunoassay, and (D) boronate affinity HPLC procedures.

Table 12. Commutability results for pairwise comparison across four measurement procedures in ten HbA_{1C} blood materials

HbA_{1C} blood materials	HbA_{1C} ranges from 5 measurement procedure	Commutability assessment across paired HbA_{1C} measurement			
		EN/ CE	EN/ CE-HPLC	EN/ TI	EN/ BA-HPLC
Processed blood materials					
FPB1	5.04 - 5.40%	C	C	C	C
SBD1	5.03 - 5.29%	C	C	C	C
FPB2	6.23 - 6.77%	C	C	C	NC (Lower 95%PI)
FPB3	7.85 - 8.56%	C	C	C	NC (Lower 95%PI)
FPB4	10.64 - 11.48%	C	C	C	NC (Lower 95%PI)
IGB1	7.70 - 11.30%	NC (Lower 95%PI)	NC (Lower 95%PI)	C	NC (Higher 95%PI)
Unprocessed native blood samples					
FWB1	5.05 - 5.20%	C	C	C	C
FWB2	6.84 - 7.03%	C	C	C	C
FWB3	9.47 - 9.91%	C	C	C	C
FWB4	10.75 - 11.12%	C	C	C	C

Abbreviations: FPB: frozen pooled blood materials; SBD: single blood donor materials; IGB: *in vitro* glycation blood materials; FWB: fresh whole blood materials; C: commutable blood materials; NC: non-commutable blood materials

Study 2: Determination of parameters that affect HbA_{1C} measurement

- **Parameters of HbA_{1C} blood materials**

In Study 1, the analysis focused on identifying changes in parameters associated with non-commutable PBMs. Hemoglobin A_{1C} blood materials were classified into two groups:

- Commutable PBMs: FWB1, FWB2, FWB3, FWB4.
- Non-commutable PBMs: FPB2, FPB3, FPB4, SBD, and IGB.

Analysis of factors influencing HbA_{1C} content in non-commutable compared to commutable HbA_{1C} blood materials

Table 13 presents the measurements of six parameters: VA, PA, HI, pH, HCT, and DC. All FPBs demonstrated non-commutability when measuring HbA_{1C} content by EN compared to BA-HPLC. This study investigated the factors interfering with HbA1C measurement in PBMs that showed non-commutability when HbA_{1C} content was measured by EN compared to BA-HPLC, including FPB1–4 and IGB. These were compared with commutable PBMs, including FWB1–4 and SBD.

The distribution of data for each parameter of HbA_{1C} blood materials was assessed using the Shapiro-Wilk test, which revealed that VA, HI (%), and pH had non-normal distributions ($p \leq 0.05$). Due to the non-normal distribution and the small sample size ($n = 5$), the Mann-Whitney U test was employed to compare differences for each parameter between groups. The results showed that PV, HI, HCT, and DC in the non-commutable group was significantly different ($p \leq 0.05$) from the commutable PBM group. However, deviations were also observed when comparing PBMs to unprocessed native blood samples (**Table 13**). Subsequent analysis focused on VA, PA, HI, pH, HCT, and DC results in PBMs compared to unprocessed native blood samples to further investigate these deviations (**Table 14**).

Table 13. Measurement of parameters in 10 BMs with various HbA_{1C} by EN measurement

HbA_{1C} blood materials	**Average ± SD of each parameter (n=3)**					
	HbA_{1C} content	**PV**	**HI (%)**	**pH**	**HCT (%)**	**DC (%)**
Commutable HbA_{1C} blood materials (EN/BA-HPLC)						
FWB1	5.09 ± 0.02	0.46 ± 0.11	10 ± 3	7.87 ± 0.07	39 ± 1	1.05 ± 0.02
FWB2	6.84 ± 6.05	0.51 ± 0.05	14 ± 5	7.83 ± 0.05	35 ± 2	2.34 ± 0.02
FWB3	9.47 ± 0.02	0.64 ± 0.21	11 ± 1	7.95 ± 0.14	39 ± 1	1.37 ± 0.13
FWB4	10.75 ± 0.01	0.67 ± 0.02	15 ± 0	8.00 ± 0.10	45 ± 0	1.32 ± 0.02
SBD	5.16 ± 0.02	3.89 ± 0.42	86 ± 9	7.16 ± 0.20	41 ± 0	2.06 ± 0.02
FPB1	5.27 ± 0.02	3.70 ± 0.62	82 ± 14	7.69 ± 0.10	0.00	1.93 ± 0.02
Min-Max		0.46-3.89	10-86	7.16-8.00	35-45	1.05-2.34
Shapiro-Wilk (*p*-value)		**0.005***	**0.005***	**0.059***	**0.004***	0.606
Mann-Whitney U test (*p*-value)		0.133	0.133	1.000	0.533	0.133
Non-commutable HbA_{1C} blood materials (EN/BA-HPLC)						
FPB2	6.58 ± 0.01	4.44 ± 0.05	99 ± 1	7.71 ± 0.09	0.00	2.22 ± 0.02
FPB3	8.22 ± 0.02	4.06 ± 0.38	90 ± 8	7.59 ± 0.08	0.00	2.29 ± 0.05
FPB4	10.00 ± 0.02	3.92 ± 0.54	87 ± 12	7.68 ± 0.07	0.00	2.47 ± 0.04
IGB	8.20 ± 0.03	4.19 ± 0.27	93 ± 6	7.00 ± 0.08	21 ± 1	2.34 ± 0.05
Min-Max		3.70-4.44	82-99	7.00-7.69	0-21	1.93-2.47
Shapiro-Wilk (*p*-value)		0.898	0.850	**0.044***	**0.001***	0.851
Mann-Whitney U test (*p*-value)		**0.010****	**0.010****	**0.114**	**0.038****	**0.038****

Abbreviations: N: no hemolysis observed; PA: plasma absorbance; VA: visual appearance; HI: hemolysis index; HCT: hematocrit; DC: debris cell count; C: commutable materials; NC: non-commutable materials.

** A significant ($p \leq 0.05$) of parameter in non-commutable materials when compared to commutable groups

Analysis of VA, PA, HI, pH, HCT, and DC results in PBMs compared to unprocessed native blood samples

Three tubes of each PBM and unprocessed native blood sample were tested for VA, PA, HI, pH, HCT, and DC. The results for each parameter in PBMs were compared to those of unprocessed native blood samples. The Shapiro-Wilk test was used to assess the data distribution in three tubes for each HbA_{1C} blood material. Results indicated that VA, HI (%), HCT (%), and pH in some HbA_{1C} blood materials did not follow a normal distribution. Due to this non-normal distribution, the Kruskal-Wallis test was employed to compare VA, PA, HI, pH, HCT, and DC results between PBMs and unprocessed native blood samples.

The analysis revealed significant differences in VA, PA, HI, pH, and DC for at least one pair. Pairwise comparisons identified specific differences, with statistically significant deviations ($p \leq 0.05$) observed in the VA of FPB2, the pH of IGB, and the DC of FPB4 compared to unprocessed native blood samples (**Table 14**). Notably, it was observed that the DC in PBMs varied according to HbA_{1C} content. Furthermore, DC was positively correlated with HbA_{1C} content ($r = 0.958$, $p \leq 0.05$) (**Table 15**).

A retrospective analysis of FPB production lots showed no significant differences ($p > 0.05$) in DC between commutable and non-commutable FPB groups. Additionally, HbA_{1C} content exceeding 10.75% and DC levels greater than 2.81% were associated with an increased risk of non-commutability (**Table 16**).

Table 14. Analysis of VA, PA, HI, pH, HCT, and DC results in PBMs compared to unprocessed native blood samples

HbA_{1C} blood materials	**N=3**	**Parameter of each HbA1C blood materials (%NGSP) (n=1)**				
		VS	**HI (%)**	**HCT (%)**	**pH**	**DC (%)**
Commutable HbA_{1C} blood materials (EN/BA-HPLC)						
FWB1	Mean ± SD	0.46 ± 0.11	10.17 ± 2.52	38.67 ± 0.58	7.87 ± 0.07	1.05 ± 0.02
	Shapiro-Wilk (*p*-value)	0.900	0.952	0.000	0.537	1.000
FWB2	Mean ± SD	0.51 ± 0.05	14.19 ± 4.65	38.67 ± 0.58	7.83 ± 0.05	1.37 ± 0.13
	Shapiro-Wilk (*p*-value)	0.637	0.075	0.637	0.612	0.637
FWB3	Mean ± SD	0.64 ± 0.21	11.41 ± 1.15	35.33 ± 1.53	7.95 ± 0.14	2.34 ± 0.02
	Shapiro-Wilk (*p*-value)	0.194	0.651	0.000	0.878	1.000
FWB4	Mean ± SD	0.67 ± 0.02	14.85 ± 0.46	45 ± 0	8 ± 0.1	1.32 ± 0.02
	Shapiro-Wilk (*p*-value)	1.000	0.708	NA	0.000	0.463
Non-commutable HbA_{1C} blood materials (EN/BA-HPLC)						
FPB1	Mean ± SD	3.7 ± 0.62	82.2 ± 13.85	0 ± 0	7.69 ± 0.1	1.93 ± 0.02
	Shapiro-Wilk (*p*-value)	0.341	0.341	NA	0.384	0.637
FPB2	Mean ± SD	4.44 ± 0.05**	98.63 ± 1.19	0 ± 0	7.71 ± 0.09	2.22 ± 0.02
	Shapiro-Wilk (*p*-value)	0.000	0.000	NA	0.756	0.463
FPB3	Mean ± SD	4.06 ± 0.38	90.2 ± 8.49	38.67 ± 0.58	7.59 ± 0.08	2.29 ± 0.05
	Shapiro-Wilk (*p*-value)	0.000	0.000	NA	0.122	0.637
FPB4	Mean ± SD	3.92 ± 0.54	87.22 ± 11.94	38.67 ± 0.58	7.68 ± 0.07	2.47 ± 0.04**
	Shapiro-Wilk (*p*-value)	0.731	0.733	0.843	0.843	
IGB	Mean ± SD	4.19 ± 0.27	93.19 ± 5.89	21.33 ± 0.58	7.00 ± 0.08**	2.34 ± 0.05
	Shapiro-Wilk (*p*-value)	0.473	0.479	NA	0.927	0.000
SBD	Mean ± SD	3.89 ± 0.42	86.46 ± 9.37	41 ± 0	7.16 ± 0.2	2.06 ± 0.02
	Shapiro-Wilk (*p*-value)	0.791	0.794	NA	0.246	1.000
10 BM	**kruskal wallis test (*p*-value)**	**0.004**	**0.004**	**NA**	**0.003**	**0.001**

Abbreviations: N: no hemolysis observed; PA: plasma absorbance; VA: visual appearance; HI: hemolysis index; HCT: hematocrit; DC: debris cell count

** A statistically significant difference ($p < 0.001$) when comparing the parameter with unprocessed native blood sample

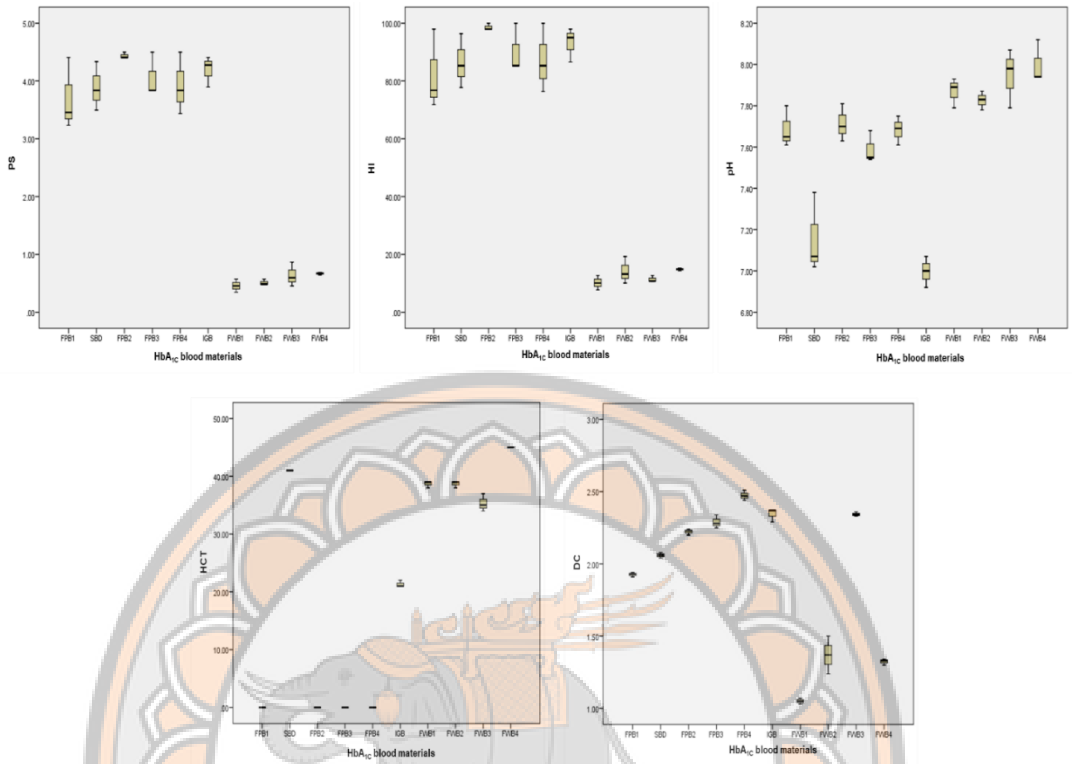


Figure 18. Graph showing the Mean ± SD results for each parameter

Table 15. The correlation between HbA_{1C} content with DC

HbA_{1C} blood materials	**Parameter**	
	HbA_{1C} content	DC
FPB1	5.27	1.93
FPB2	6.58	2.22
FPB3	8.22	2.29
FPB4	10.00	2.47
IGB1	8.20	2.34
SBD1	5.16	2.06
FWB1	5.09	1.05
FWB2	6.84	2.34
FWB3	9.47	1.37
FWB4	10.75	1.32
Pearson Correlation (*p*-value)	r=0.958 (p <0.05))	

The study revealed that debris cell count significantly impacts the commutability of FPB. The researcher analyzed the debris cell count from 10 samples produced at different time intervals and observed variations in debris cell count across production batches.

The commutability evaluation of FPB, measured between the EN/ BA-HPLC procedures, indicated non-commutability when the debris cell count was high. The findings are presented in **Table 16**, and the commutability analysis is illustrated using Deming regression plots in **Figure 19**.

Table 16. Derbies cell count analysis and commutability assessment paired EN/BA-HPLC procedures in ten FPB

HbA_{1C} blood materials	**HbA1C (%NGSP)**			**Parameter measurement**	**Commutability (EN/BA-HPLC)**
	Target value	**EN**	**BA-HPLC**	**DC**	
FPB12	10.39	10.7	10.2	3.32	NC
FPB14	11.03	11.3	10.7	2.47	NC
Mean± SD				2.90±0.06	
FPB5	5.43	5.20	5.30	1.71	C
FPB6	5.54	5.60	5.50	2.58	C
FPB10	6.32	6.40	6.20	1.89	C
FPB7	6.44	6.30	6.70	1.81	C
FPB8	6.62	6.60	6.50	2.22	C
FPB11	7.13	7.20	7.00	2.81	C
FPB9	7.37	7.20	7.30	1.81	C
FPB13	10.75	11.00	10.90	1.93	C
Mean± SD				2.10±0.40	
Mann-Whitney U test (*p*-value)				0.116	

Abbreviations: C: commutable blood materials; NC: non-commutable blood material

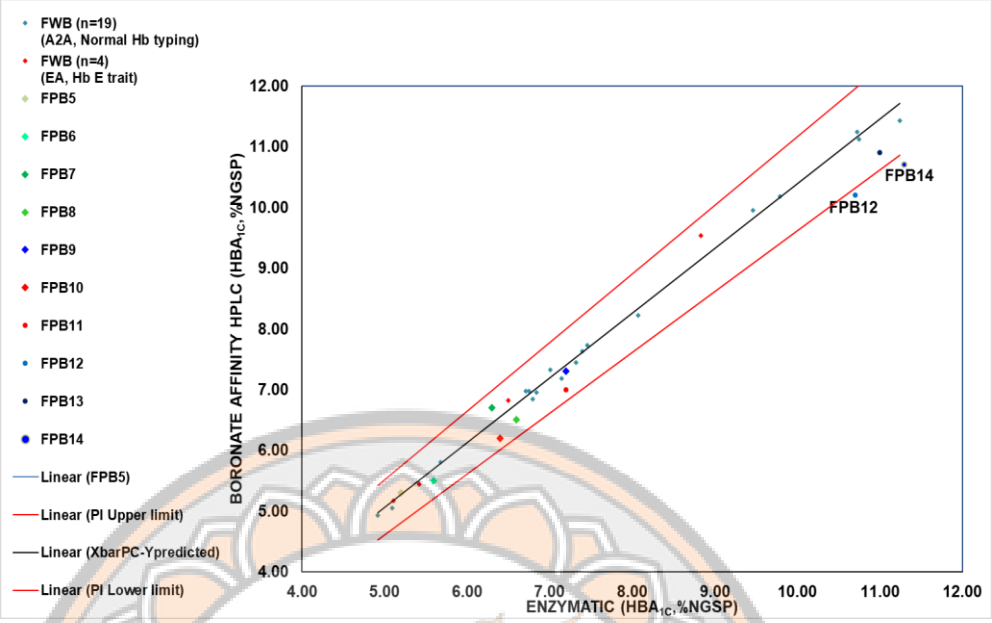


Figure 19. Deming regression plots comparing enzymatic assay with boronate affinity HPLC procedures. Abbreviations: FPB: frozen pooled blood materials.

Study 3: Adjust the parameters of the blood materials that affect HbA_{1C} measurement

The study demonstrated that PBMs exhibited increased HI and red blood cell hemolysis, resulting in HCT levels lower than those of unprocessed blood samples. Additionally, the pH of FBMs ranged from 7.68 to 7.71, slightly lower than the range observed in unprocessed native blood samples (7.83–8.00). Although these parameters did not directly affect the commutability of PBMs, adjustments should be made during production to address these variations.

PBMs produced through pooled blood methods allow for the production of large quantities with extended stability. However, a limitation of this material is the inability to fully prevent blood hemolysis during production. Regarding debris cell count, which is highly likely to affect HbA_{1C} measurement, its levels should be minimized and controlled within an acceptable range not exceeding 2.08%.

Study 4: Assess of homogeneity and stability of HbA_{1C} blood materials

1. Homogeneity test of processed blood materials

Frozen pooled blood materials underwent homogeneity testing to confirm their suitability for laboratory analysis. The FPBs were prepared from remnant EDTA blood and categorized into specific HbA_{1C} content ranges: FPB1 (5.0-5.7%), FPB2 (6.0-7.0%), FPB3 (8.0-9.0%), and FPB4 (10.0-12.0%), with storage at -70°C

Homogeneity was assessed by randomly selecting 10 vials from each PBM batch, followed by HbA_{1C} content analysis using one-way ANOVA. Results indicated that the $F_{calculation}$ values for all PBMs were less than the $F_{critical}$ value (3.020), confirming the homogeneity of each material. Detailed analysis results are presented in **Tables 17–20**, with a summary provided in **Table 21**. These findings establish the uniformity and reliability of the PBMs, ensuring their suitability for subsequent analyses and validating their use in laboratory and research applications.

Table 17. Analysis of FPB1 data using One-Way ANOVA (Single Factor)

Groups	Count	Sum	Average	Variance		
1	2	10.22	5.11	0.0002		
2	2	10.24	5.12	0.0002		
3	2	10.21	5.105	5E-05		
4	2	10.23	5.115	5E-05		
5	2	10.25	5.125	5E-05		
6	2	10.26	5.13	0.0002		
7	2	10.29	5.145	0.00045		
8	2	10.22	5.11	0.0002		
9	2	10.23	5.115	5E-05		
10	2	10.23	5.115	0.00125		
Source of Variation	SS	df	MS	F	*p*-value	F_{crit}
Between Groups	0.00248	9	0.000276	1.021	0.483213	3.020383
Within Groups	0.0027	10	0.00027			
Total	0.00518	19				

Table 18. Analysis of FPB2 data using One-Way ANOVA (Single Factor)

Groups	Count	Sum	Average	Variance
1	2	13.02	6.51	0.0002
2	2	12.98	6.49	0.0002
3	2	12.97	6.485	5E-05
4	2	12.98	6.49	0
5	2	13	6.5	0
6	2	13.02	6.51	0.0002
7	2	12.99	6.495	5E-05
8	2	12.95	6.475	0.00045
9	2	12.99	6.495	5E-05
10	2	13	6.5	0

Source of Variation	SS	df	MS	F	*p*-value	F_{crit}
Between Groups	0.0021	9	0.000233	1.944	0.157386	3.020383
Within Groups	0.0012	10	0.00012			
Total	0.0033	19				

Table 19. Analysis of FPB3 data using One-Way ANOVA (Single Factor)

Groups	Count	Sum	Average	Variance
1	2	16.64	8.32	0.0002
2	2	16.68	8.34	0
3	2	16.67	8.335	0.00045
4	2	16.68	8.34	0.0002
5	2	16.67	8.335	5E-05
6	2	16.69	8.345	5E-05
7	2	16.66	8.33	0.0002
8	2	16.66	8.33	0.0002
9	2	16.69	8.345	5E-05
10	2	16.68	8.34	0.0002

Source of Variation	SS	df	MS	F	*p*-value	F_{crit}
Between Groups	0.00108	9	0.00012	0.750	0.661992	3.020
Within Groups	0.0016	10	0.00016			
Total	0.00268	19				

Table 20. Analysis of FPB4 data using One-Way ANOVA (Single Factor)

Groups	Count	Sum	Average	Variance
1	2	22.49	11.245	0.00045
2	2	22.46	11.23	0
3	2	22.4	11.2	0.0002
4	2	22.46	11.23	0
5	2	22.43	11.215	0.00045
6	2	22.36	11.18	0.0032
7	2	22.46	11.23	0
8	2	22.41	11.205	0.00045
9	2	22.41	11.205	0.00125
10	2	22.44	11.22	0

Source of Variation	SS	df	MS	F	*p*-value	F_{crit}
Between Groups	0.00648	9	0.00072	1.200	0.387781	3.020383
Within Groups	0.006	10	0.0006			
Total	0.01248	19				

Table 21. Homogeneity test of four FPMs

NO	FPB1 (HbA_{1C} (%NGSP)		FPB2 (HbA_{1C} (%NGSP)		FPB3 (HbA_{1C} (%NGSP)		FPB4 (HbA_{1C} (%NGSP)	
	Resilt 1	Rusult 2	Rusult 1	Rusult 2	Rusult 1	Rusult 2	Rusult 1	Rusult 2
1	5.12	5.10	6.52	6.50	8.31	8.33	11.26	11.23
2	5.11	5.13	6.50	6.48	8.34	8.34	11.23	11.23
3	5.10	5.11	6.48	6.49	8.35	8.32	11.21	11.19
4	5.11	5.12	6.49	6.49	8.33	8.35	11.23	11.23
5	5.12	5.13	6.50	6.50	8.34	8.33	11.23	11.20
6	5.12	5.14	6.52	6.50	8.35	8.34	11.22	11.14
7	5.13	5.16	6.50	6.49	8.34	8.32	11.23	11.23
8	5.12	5.10	6.46	6.49	8.34	8.32	11.22	11.19
9	5.11	5.12	6.50	6.49	8.35	8.34	11.18	11.23
10	5.09	5.14	6.50	6.50	8.35	8.33	11.22	11.22
Analyzer	Alinity c		Alinity c		Alinity c		Alinity c	
Mean	5.12		6.50		8.34		11.22	
SD	0.02		0.01		0.01		0.03	
$F_{calculation}$	1.021		1.944		0.750		1.200	
$F_{critical}$	3.020		3.020		3.020		3.020	
Summary	$F_{cal} < F_{cri}$ Homogeneous		$F_{cal} < F_{cri}$ Homogeneous		$F_{cal} < F_{cri}$ Homogeneous		$F_{cal} < F_{cri}$ Homogeneous	

Abbreviations: FPB: frozen pooled blood materials

2. Stability test of processed blood materials

The stability of frozen pooled blood materials (FPBs) was evaluated under storage at -70°C for varying durations. The study demonstrated that FPB1–4 remained stable, with $T_{cal} < T_{critical}$ across all tested time points (0-month, 3-month, 6 month, 9 month, and 12 month), as analyzed based on ISO Guide 35 statistical criteria. This finding indicates that FPBs can be reliably stored at -70°C for up to 12 months without compromising their stability.

The stability of HbA_{1C} content in FPBs was measured as HbA_{1C} (%NGSP) using the Alinity c analyzer. The results are summarized in **Table 22**, showing consistent HbA_{1C} measurements over the storage period. Statistical analysis confirmed that all four FPBs met the stability criteria, with calculated T values (T_{cal}) lower than critical T values ($T_{critical}$).

Table 22. Stability test of four FPMs

Month	**FPB1 (HbA_{1C} (%NGSP))**	**FPB2 (HbA_{1C} (%NGSP))**	**FPB3 (HbA_{1C} (%NGSP))**	**FPB4 (HbA_{1C} (%NGSP))**
0	5.12	6.50	8.34	11.22
3	5.65	6.62	8.40	11.39
6	5.26	6.64	8.43	11.30
9	5.60	6.52	8.78	11.30
12	5.27	6.58	8.22	11.00
Analyzer	Alinity c	Alinity c	Alinity c	Alinity c
Mean	5.38	6.57	8.43	11.24
T_{cal}	0.306	0.295	0.190	1.161
$T_{critical}$	3.182	3.182	3.182	3.182
Summary	$t_{cal} < t_{critical}$ Stable	$t_{cal} < t_{critical}$ Stable	$t_{cal} < t_{critical}$ Stable	$t_{cal} < t_{critical}$ Stable

Abbreviations: FPB: frozen pooled blood materials

Discussion

Accurate measurement of HbA_{1C} is essential for effective diabetes mellitus management, as it ensures that clinical decisions are appropriately aligned with patients' glycaemic status. Blood-based reference materials play a fundamental role in harmonizing HbA_{1C} measurements among laboratories by enabling traceability to primary reference measurement procedures. In Thailand, where multiple analytical platforms are used for HbA_{1C} determination, the availability of commutable materials is particularly important for assessing both accuracy and inter-method comparability.

The Department of Medical Sciences, for instance, incorporates fresh blood materials (FBMs) into its external quality assessment (EQA) schemes (Suparak et al., 2022). These materials are assigned target values traceable to the IFCC secondary reference measurement procedures, thereby maintaining consistency with international standards. Likewise, the Royal College of Pathologists of Australasia Quality Assurance Programs (RCPAQAP) utilizes fresh whole blood (FWB) and lyophilized blood materials in EQA programs across Australia, New Zealand, Malaysia, Hong Kong, and Singapore. However, the RCPAQAP program is not recommended for laboratories using the Abbott Affinion system, for which participation in the FWB program is advised (RCPAQAP, 2023). In addition, the $EurA_{1C}$ program employs both FWB and lyophilized materials to assess laboratory performance in Asia, Europe, and Africa (IFCC, 2021). Collectively, these programs underscore the importance of commutable materials in interlaboratory comparison and standardization, highlighting the need for systematic commutability evaluations across diverse analytical methods.

This study assessed the commutability of PBMs, including FPBs, SBD, and IGB. The EN procedure was used as the reference measurement procedures (RMP) for comparison with other HbA_{1C} measurement procedures in this study. The Δ_m between the EN procedure and the IFCC secondary RMP was within the acceptable combined uncertainty ($\Delta_m \leq U_\Delta$). These findings confirm the traceability of the EN procedure to the IFCC standard, reinforcing its reliability for HbA_{1C} measurement. The HbA_{1C} content of 19 cases of normal Hb typing from CBSs, measured using the RMP (EN procedure), showed significant differences ($p < 0.05$) across four

measurement procedures: CE, CE-HPLC, TI, and BA-HPLC. This suggests that variations in measurement methods may influence HbA_{1C} quantification, and potential analytical variability may be associated with the limited sample size.

HbA_{1C} concentrations in 23 CBSs were subsequently measured using the RMP and the four analytical methods, and Deming regression was applied to each paired comparison. The lambda (λ) values, defined as the ratio of measurement error variances between the test methods (Y-axis) and the RMP (X-axis), were greater than 1.00. This indicates that the RMP exhibited lower measurement error variance compared with CE, CE-HPLC, TI, and BA-HPLC (Carroll, 1998).

Commutability assessment showed that FPB1 and SBD were commutable across all four paired measurement procedures. FPB2 and FPB3 were commutable when evaluated using RMP-TI, RMP-CE, and RMP-CE-HPLC, but were non-commutable with RMP-BA-HPLC. In contrast, IGB was commutable only when assessed using RMP-TI, consistent with previous reports (W Duanginta et al., 2023; Wanutchaya Duanginta, Apiratmateekul, Tran, Nammoonnoy, & Treebuphachatsakul, 2024), and was non-commutable when compared using CE, CE-HPLC, and BA-HPLC.

Matrix-related alterations in PBMs were initially examined through visual assessment of plasma appearance. PBMs displayed altered plasma coloration relative to unprocessed native blood. Further analysis revealed that plasma absorbance and hemolysis index (HI) were higher in non-commutable PBMs than in commutable ones. Additionally, hematocrit (HCT) levels were lower in non-commutable PBMs. These differences were attributed primarily to hemolysis induced by freeze-processing. Although previous studies have reported interference in HbA_{1C} measurement from frozen blood compared with fresh whole blood (Liotta, Di Franco, Pazzagli, & Luconi, 2013), other investigations have shown that storage at −80 °C does not affect HbA_{1C} results obtained by CE-HPLC (Bergmann & Sypniewska, 2016; Jones et al., 2004; Rolandsson, Marklund, Norberg, Ågren, & Hägg, 2004). In the present study, commutability was evaluated according to CLSI EP4 guidelines. FPBs were found to be commutable for HbA_{1C} measurements when comparing RMP with CE, CE-HPLC, and TI. However, non-commutability was observed for RMP-BA-HPLC when HbA_{1C} concentrations exceeded 6.23% NGSP. This may be

attributable to increased sample turbidity interfering with light absorption during BA-HPLC analysis, thereby affecting result comparability.

The influence of HCT on HbA_{1C} measurement remains inconclusive. Notably, FPB1, which had an HCT of 0%, was commutable across all analytical methods. Nevertheless, extremely low HCT values may pose challenges for certain methodologies, warranting careful interpretation.

pH levels in the commutable group (pH 7.16–7.69) were higher than those in the non-commutable (pH 7.00) when measuring HbA_{1C} content between RMP and BA-HPLC. Agilent Technologies highlighted the significant influence of pH on the separation of ionizable compounds in HPLC analysis (2015). Previous research also suggests that pH affects boronate interactions in solutions; however, the exact mechanism remains unclear (António, Russo, Carvalho, Cal, & Gois, 2019; Springsteen & Wang, 2002; Wu et al., 2013). It is plausible that pH influences the binding between cis-diol groups and glycated Hb in the BA-HPLC procedure, where optimal interaction typically occurs at a pH of 8.0 (Klenk et al., 1982).

The DC analysis revealed the presence of an FA peak in FPB, which may have resulted from prolonged blood storage, potentially leading to sample degradation and the appearance of unidentifiable peaks in the chromatogram. Additionally, an increased P0 peak was observed in PBM chromatograms. These chromatographic changes impacted the calculation of $\%HbA_{1C}$ using the CE-HPLC. According to the laboratory procedure manual, if unidentifiable peaks (FA and P0) are observed, the cause should be investigated before reporting the test results (Little, 2019). However, this study demonstrated that FPB remained commutable when measuring HbA_{1C} content between RMP and CE-HPLC. For HbA_{1C} measurement using BA-HPLC, a highly specific method, DC was not identified as an interfering factor in non-commutable blood materials.

CHAPTER V

CONCLUSIONS

This study demonstrated that increases in visual appearance (VA), plasma absorbance (PA), and hemolysis index (HI) resulting from blood hemolysis did not significantly affect HbA_{1C} measurements when analyzed using capillary electrophoresis (CE), CE-HPLC, and turbidimetric immunoassay (TI), thereby preserving sample commutability across these analytical platforms. In contrast, these hemolysis-related factors were found to adversely influence HbA_{1C} results obtained by boronate affinity high-performance liquid chromatography (BA-HPLC), particularly when HbA_{1C} concentrations exceeded 6.23% NGSP. Therefore, when fresh processed blood (FPBs) is employed as external quality assessment (EQA) materials, specific limitations associated with BA-HPLC-based HbA_{1C} measurement must be carefully considered.

Although hematocrit (HCT) could not be conclusively identified as a primary determinant of non-commutability, maintaining HCT levels comparable to those of unprocessed whole blood is recommended to enhance compatibility across different analytical systems. In addition, the detection of unidentified chromatographic peaks—likely attributable to increased hemoglobin degradation products resulting from prolonged FPB storage—warrants careful monitoring, as such interference may contribute to inaccuracies in HbA1c measurement.

Overall, the findings of this study highlight the importance of controlling pre-analytical variables and selecting appropriate analytical methods when developing and implementing FPB-based EQA materials, particularly for laboratories employing BA-HPLC systems. These considerations are essential to ensure reliable HbA_{1C} measurement, method harmonization, and the continued comparability of results across different platforms.

REFERENCES

AmericanDiabetesAssociation. (2023). Retrieved from https://diabetes.org/diabetes/a1c/diagnosis

AmericanDiabetesAssociation. (2021). 2. Classification and diagnosis of diabetes: standards of medical care in diabetes—2021. *Diabetes care, 44*(Supplement_1), S15-S33.

António, J. P., Russo, R., Carvalho, C. P., Cal, P. M., & Gois, P. M. (2019). Boronic acids as building blocks for the construction of therapeutically useful bioconjugates. *Chemical Society Reviews, 48*(13), 3513-3536.

Atlas, I. D. (2023, 2023). Diabetes around the world in 2021. Retrieved from https://diabetesatlas.org/

Bergmann, K., & Sypniewska, G. (2016). The influence of sample freezing at-80° C for 2-12 weeks on glycated haemoglobin (HbA1c) concentration assayed by HPLC method on Bio-Rad D-10© auto-analyzer. *Biochemia Medica, 26*(3), 346-352.

CAP. (2024, May 2024). College of American Pathologists (CAP) GH5 Survey Data: (updated 5/24). Retrieved from https://ngsp.org/CAP/CAP24a.pdf

Carroll, R. J. (1998). Measurement error in epidemiologic studies. *Encyclopedia of biostatistics, 3*, 2491-2519.

Corporation, K. R&D Success: Enzyme assay for HbA1c. Retrieved from https://www.kikkoman.com/en/quality/research/story/02.html

d'Almeida, M., Jagger, J., Duggan, M., White, M., Ellis, C., & Chin-Yee, I. (2000). A comparison of biochemical and functional alterations of rat and human erythrocytes stored in CPDA-1 for 29 days: implications for animal models of transfusion. *Transfusion Medicine, 10*(4), 291-303.

David Warmflash, M., Nathan H Lents, Ph.D. (2016). Blood Biology I: Components of Blood. *https://www.visionlearning.com/es/library/Biologia/2/Blood-Biology-I/242*.

DiabetesAssociationofThailand. (2018). *Clinical Practice Guideline for Diabetes 2017* (Vol. 220). Romyen Media Co., Ltd.

Duanginta, W., Apiratmateekul, N., Kost, G., Tran, N., Kongros, K., Shearman, K., & Treebuphachatsakul, W. (2023). Challenges of In Vitro Glycation when Producing Blood Materials for Hemoglobin A1C Immunoassays. *Clinical Laboratory, 69*(4).

Duanginta, W., Apiratmateekul, N., Tran, N. K., Nammoonnoy, J., & Treebuphachatsakul, W. (2024). Commutability and Uncertainty of Blood Hemoglobin A1c Testing Materials. *Clinical Laboratory, 70*(3).

Ellison, S. L., & Botha, A. (2018). Principles for the assessment of homogeneity and stability in the new ISO Guide 35: 2017. *Accreditation and Quality Assurance, 23*(1), 47-51.

Freeman, V. S. (2014). Glucose and hemoglobin A1c. *Laboratory Medicine, 45*(1), e21-e24.

Gupta, S., Jain, U., & Chauhan, N. (2017). Laboratory diagnosis of HbA1c: a review. *J Nanomed Res, 5*(4), 00120.

Hörber, S., Achenbach, P., Schleicher, E., & Peter, A. (2020). Harmonization of immunoassays for biomarkers in diabetes mellitus. *Biotechnology advances, 39*, 107359.

IFCC. (2021, 3 Mar 2025). EurA1c trial. Retrieved from http://www.ifcchba1c.net/GeneralInformation.aspx?I=17

Jones, W., Scott, J., Leary, S., Stratton, F., Smith, S., Jones, R., . . . Team, A. S. (2004). Stability of whole blood at− 70° C for measurement of hemoglobin A1c in healthy individuals. *Clinical chemistry, 50*(12), 2460-2461.

Klenk, D. C., Hermanson, G. T., Krohn, R. I., Fujimoto, E. K., Mallia, A. K., Smith, P., . . . Goldstein, D. (1982). Determination of glycosylated hemoglobin by affinity chromatography: comparison with colorimetric and ion-exchange methods, and effects of common interferences. *Clinical chemistry, 28*(10), 2088-2094.

Lab, W. M. (2024). Proficiency Testing Program for Hemoglobin A1c.

Liotta, L., Di Franco, A., Pazzagli, M., & Luconi, M. (2013). Glycated hemoglobin (HbA1c) measurement in frozen whole blood depends on baseline values of fresh samples. *Analytical and bioanalytical chemistry, 405*, 429-434.

Little, D. R. (2019). Laboratory Procedure Manual for Tosoh G8 Glycohemoglobin Analyzer. Retrieved from tps://wwwn.cdc.gov/nchs/data/nhanes/public/2017/labmethods/GHB-J-G8-508.pdf

Miller, W. G., Schimmel, H., Rej, R., Greenberg, N., Ceriotti, F., Burns, C., . . . Nilsson, G. (2018). IFCC working group recommendations for assessing commutability part 1: general experimental design. *Clinical chemistry, 64*(3), 447-454.

Ministry of Public Health, T. (2023). Number and mortality rate of non-communicable diseases (years 2018-2022). Retrieved from https://ddc.moph.go.th/dncd/forecast_detail.php?publish=13744. from Ministry of Public Health https://ddc.moph.go.th/dncd/forecast_detail.php?publish=13744

Mohammadi, R., & Norozi, V. (2016). HbA1c External Quality Assessment: Commutable vs Noncommutable Samples. *Biomedical and Pharmacology Journal, 9*(1), 163-168.

NGSP. Convert between NGSP, IFCC and eAG units. Retrieved from https://ngsp.org/convert1.asp

NGSP. (2010). IFCC Standardization of HbA1c. Retrieved from https://ngsp.org/ifcc.asp

NGSP. (2023). List of NGSP Certified Laboratories. Retrieved from chrome-extension://efaidnbmnnnibpcajpcglclefindmkaj/https://ngsp.org/docs/labs.pdf

RCPAQAP. (2023). HbA1c: Lyophilised and HbA1c: Whole Blood. Retrieved from https://rcpaqap.com.au/?s=HbA1c&post_type=product

Reverberi, R., & Reverberi, L. (2007). Factors affecting the antigen-antibody reaction. *Blood transfusion, 5*(4), 227.

Rhea, J. M., & Molinaro, R. (2014). Pathology consultation on HbA1c methods and interferences. *American journal of clinical pathology, 141*(1), 5-16.

Rolandsson, O., Marklund, S. L., Norberg, M., Ågren, Å., & Hägg, E. (2004). Hemoglobin A1c can be analyzed in blood kept frozen at− 80 C and is not commonly affected by hemolysis in the general population. *Metabolism, 53*(11), 1496-1499.

Sacks, D. B. (2012). Measurement of hemoglobin A1c: a new twist on the path to harmony. *Diabetes care, 35*(12), 2674-2680.

Springsteen, G., & Wang, B. (2002). A detailed examination of boronic acid–diol complexation. *Tetrahedron, 58*(26), 5291-5300.

Suparak, S., Pratumvinit, B., Ngueanchanthong, K., Unpol, P., Thanomsakyuth, A., Setthaudom, C., . . . Uppapong, B. (2022). External quality assessment scheme for HbA1c assays in Thailand: A 5-year experience. *Practical Laboratory Medicine, 31*, e00288.

Technologies, A. (2015). Control pH During Method Development for Better Chromatography. Retrieved from https://wemedlab.com/?ae_global_templates=eqa3

Trapmann, S., Botha, A., Linsinger, T. P., Mac Curtain, S., & Emons, H. (2017). The new International Standard ISO 17034: general requirements for the competence of reference material producers. *Accreditation and Quality Assurance, 22*(6), 381-387.

TrinityBiotech. (2023). Boronate Affinity Chromatography. Retrieved from https://www.trinitybiotech.com/haemoglobins/boronate-affinity-chromatography/

V., P. (2016). *nterference of hemoglobin variants in HbA1c measurement using ion-exchange chromatography.* (Master of Medical Technology). Thammasat University,

Weykamp, C. (2013). HbA1c: a review of analytical and clinical aspects. *Annals of laboratory medicine, 33*(6), 393.

Wongsri, P. (2018). *Preparation qualty control materials for hemoglobin A1c testing from blood samples.*

Wu, X., Li, Z., Chen, X.-X., Fossey, J. S., James, T. D., & Jiang, Y.-B. (2013). Selective sensing of saccharides using simple boronic acids and their aggregates. *Chemical Society Reviews, 42*(20), 8032-8048.