

Laboratory Procedure Manual

Analyte: **Glycohemoglobin**

Matrix: **Whole Blood**

Method **Tosoh G 7 HPLC
Glycohemoglobin Analyzer**

as performed by: **Fairview-University Medical Center
University Campus
Collaborative Studies Clinical Laboratory
Minneapolis, Minnesota**

Contact: Dr. Michael Steffes

Important Information for Users

The Fairview -University Medical Center periodically refines these laboratory methods. It is the responsibility of the user to contact the person listed on the title page of each write-up before using the analytical method to find out whether any changes have been made and what revisions, if any, have been incorporated.

Public Release Data Set Information

This document details the Lab Protocol for testing the items listed in the following table:

File Name	Variable Name	SAS Label
GHB_E	LBXGH	Glycohemoglobin (%)

There was an upgrade in instruments in June 2007. During the first six months of 2007 the laboratory used Tosoh 2.2 Plus and from the second six months forward the Tosoh G7 Automated HPLC System Glycohemoglobin Analyzer was used. The method used in the first half of 2007 is described in a separate document

1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

A. Principle

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

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

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

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

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

B. Clinical Relevance

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

Although a fasting blood glucose measurement gives the clinician information about the patient's status over the last twelve hours, the stable HbA1c offers a more

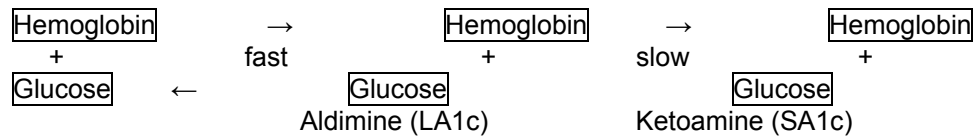
accurate indication of the patient's long-term diabetic control over the last two to three months.

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

HbA1c is nonenzymatically synthesized in two steps:

1. The glucose aldehyde group and the free amino group on the valine in the N-terminus of the hemoglobin beta chain react to form the Schiff base, aldimine (also known as labile HbA1c or LA1c).
2. A stable ketoamine form of the hemoglobin complex (SA1c) is then produced by a reaction known as Amadori rearrangement.

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



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

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

2. SAFETY PRECAUTIONS

Follow all procedures and policies in the Fairview-University Medical Center Laboratory Safety Manual. Consider all specimens as potentially infectious.

Sodium azide can react with copper and lead plumbing to form explosive metal azides. On disposal, flush reagents with a large volume of water to prevent the buildup of azides.

3. COMPUTERIZATION; DATA SYSTEM MANAGEMENT

NHANES SA1c% results are entered into a spreadsheet provided electronically by WESTAT, Inc for NHANES.

To access the spreadsheet click on My Computer → Z drive → User → Dep Labs → Collab Studies → NHANES → Glyhb 004.

Choose the file named with the corresponding box number.

Enter the analysis date, run number, technologist's initials, SA1c%, and result comment code.

The spreadsheet will be sent electronically by the contact person.

4. SPECIMEN COLLECTION, STORAGE, AND HANDLING PROCEDURES; CRITERIA FOR SPECIMEN REJECTION

Samples are collected and processed in mobile examination centers according to NHANES protocols.

Specimens are packaged and shipped on cold packs or dry ice according to the established schedule.

Specimens are shipped via Federal Express for delivery directly to Collaborative Studies Clinical Laboratory.

Shipments for NHANES will arrive on Tuesdays and/or Wednesdays. The shipments will consist of two boxes, one with frozen gel packs containing HbA1c specimens and one with dry ice containing frozen glucose and insulin specimens. These shipments will be recorded on the shipping log located in a blue 3 ring binder labeled NHANES Shipping Log in the receiving area.

Included in the shipping box for HbA1c (glycohem) specimens are a shipping manifest, a Federal Express airbill for return shipment, frozen gel packs, and a box or boxes of HbA1c(glycohem) specimens (vessel/vial number 004). Record the appropriate information on the shipping log. Check the specimen numbers in the box against the manifest. Write the received date on top of the box. Bring the specimens to the HbA1c desk. File the manifest in the blue 3 ring binder labeled NHANES Shipping Manifests located in the receiving area. Remove all labels from the shipping box and attach the provided airbill for return shipment. Weigh the boxes on the scale in L237 to complete the information on the airbill. Bring the boxes to the Fairview dock.

A venous whole blood specimen collected in EDTA is required. Tubes containing heparin, potassium oxalate or sodium fluoride are acceptable. Whole blood specimens are stable up to fourteen days stored at 2-8°C or up to eight hours at room temperature before analysis. Prior to analysis, mix each patient specimen by gentle inversion to ensure homogeneity.

Fingerstick capillary specimens collected using the Bio-Rad Sample Preparation Kit are an acceptable alternative to venous whole blood collection and provide enhanced stability during sample storage and transportation. Samples prepared as directed are stable for 2 weeks stored at room temperature or four weeks stored at 2-8°C.

Optimum sample volume: 1 mL whole blood
Minimum sample volume: 50 uL whole blood (for specimens of volume less than 1 mL whole blood, a manual pre-dilution (1:250) must be prepared)

5. Procedures for Microscopic Examinations

Not applicable for this procedure.

6. EQUIPMENT AND INSTRUMENTATION, MATERIALS, REAGENT PREPARATION, CALIBRATORS (STANDARDS), AND CONTROLS

Glycohemoglobin in Whole Blood using Tosoh G 7 HPLC
NHANES 2007-2008

A. Instrumentation

- (1) A1c G7 HPLC Glycohemoglobin Analyzer. Part # 019327, with 90 sample loader, Par # 019475. Tosoh Medics, Inc., 347 Oyster Pt. Blvd., Suite 201, So. San Francisco, Ca 94080.
- (2) Labquake Rotator. Catalog no. 415-110, Labindustries, Inc., 620 Hearst Avenue, Berkeley, CA 94710-1992.
- (3) Auto Dilutor, model AD-7, Catalog No. 196-7393, Bio-Rad clinical Division, 4000 Alfred Noble Drive, Hercules, CA 94547.

B. Materials

- (1) TSKgel Glyco HSi Variant Column. Part # 019680, Tosoh Medics, Inc. Guaranteed for 2500 counts; replace as necessary (as indicated by appearance of chromatograms). Stable indefinitely when stored at 4-15°C away from direct sunlight. Use only with column-matched buffers (first letter of buffer lot must match last letter of column lot). When a new column is installed, analyze 5 duplicates after calibrating and analyzing controls. Also record the previous results on the protocol page. The results must agree within established duplicate range.
- (2) Filter element, 5/pkg. Part # 019506, Tosoh Medics, Inc. Replace at or before 400 injections (do not exceed 400 injections) or when pressure rises above 150 kg/cm² (15 Mpa).
- (3) Thermal paper for A1c G 7, 10 roll/box. Part # 019563. Tosoh Medics, Inc.
- (4) Sustaining tube, Dilution Sample, 10 mL, 50/pkg, Tosoh Medics, Inc.
- (5) Adapter Ring, Dilution Sample, 5/pkg, Tosoh Medics, Inc.
- (6) Adapter Ring, Sample Rack: 12 mm, 13 mm, and 14 mm .
- (7) Sample Vials, 100/pkg. Tosoh Medics, Inc. (For preparing dilutions of whole blood samples.)
- (8) Microcentrifuge tubes, 0.6 mL polypropylene (with caps), 500/bag, Stock # CX15559. University Stores. (For preparing aliquots of controls for frozen storage.)
- (9) DIAMAT HbA1c Sample Preparation Kit, Cat. No. 196-1026, Bio-Rad Laboratories, Clinical Division, 4000 Alfred Nobel Drive, Hercules, CA 94547. Samples prepared as directed in the Instruction Manual are stable for 2 weeks at room temperature or 4 weeks at 2-8°C. Includes supplies sufficient for 100 test samples:

Sample Preparation Vials, 100/kit, each contain 1 mL of an aqueous solution of EDTA and potassium cyanide (0.25 mmol/L). Store at 5-30°C.

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Capillaries, one glass dispenser vial containing 100 sodium-heparinized capillary tubes (5 uL). Reorder box of 20 vials (50 capillary tubes/vial), Cat. No. 195-1053, Bio-Rad Laboratories, Clinical Division.

Capillary tube holder, one holder for manipulating 5 uL capillary tubes. Reorder box of 20 holders, Cat. No. 196-1054. Bio-Rad Laboratories, Clinical Division.

Labels, 4 sheets of 25 blank labels each.

Instruction Manual.

- (10) Supply Line Filters for Buffer Lines. Part#018723(1/pkg).
- (11) Sampling Needle – Std. Part#019500(1 each).
- (12) Sampling Needle – Side hole. Part#019791(1 each)
- (13) HLC-723RP- G7 Reporting Software. Part#021209(1 each)

C. Reagent Preparation

- (1) Elution Buffer HSi Variant No. 1, (S) Part # 019552 (1 x 800 mL). Tosoh Medics, Inc. Succinic acid buffer, contains less than 0.06% sodium azide as a preservative. Unopened buffer is stable until expiration date printed on label. Once open, (S) buffer is stable for three months. Store at 4-25°C. Use only with other column-matched buffers (first letter of buffer lot matches last letter of column lot). When a new lot number of buffer is installed, analyze 5 duplicate samples at the beginning of the run. Record the previous results on the protocol page also. The results must agree within the established duplicate range.
- (2) Elution Buffer HSi Variant No. 2, (S) Part # 019553 (1 x 800 mL). Tosoh Medics, Inc. Succinic acid buffer, contains less than 0.06% sodium azide as a preservative. Unopened buffer is stable until expiration date printed on label. Once open, (S) buffer is stable for three months. Store at 4-25°C. Use only with other column-matched buffers (first letter of buffer lot matches last letter of column lot). When a new lot number of buffer is installed, analyze 5 duplicate samples at the beginning of the run. Record the previous results on the protocol page also. The results must agree within the established duplicate range.
- (3) Elution Buffer Hsi Variant No. 3, (S) Part # 019554 (1 x 800 mL). Tosoh Medics, Inc. Succinic acid buffer, contains less than 0.06% sodium azide as a preservative. Unopened buffer is stable until expiration date printed on label. Once open, (S) buffer is stable for three months. Store at 4-25°C. Use only with other column-matched buffers (first letter of buffer lot matches last letter of column lot). When a new lot number of buffer is installed, analyze 5 duplicate samples at the beginning of the run. Record the previous results on the protocol page also. The results must agree within the established duplicate range.
- (4) Elution Buffer His Variant No. 1, Part # 021446 (1 x 1500 mL). Tosoh Medics, Inc.

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- (4) Hemolysis & Wash Solution, (S) Part # 018431 (1 x 800 mL. Tosoh Medics, Inc. Contains deionized water, EDTA and Triton X and contains less than 0.12% sodium azide as a preservative. Once open, (S) buffer is stable for three months. Store at 4-25°C.

D. Standards Preparation

HbA1c Calibrator Set: Calibrator 1 (5 x 4 mL) and Calibrator 2 (5 x 4 mL). Part # 018767, Tosoh Medics, Inc. Buffered human red blood cells, 2 mg/mL human hemoglobin, and 0.5 mM EDTA as preservative. Un-reconstituted calibrator set is stable stored at 4-8°C until expiration date printed on label.

- (1) Remove caps from the calibrator vials.
- (2) Reconstitute Calibrators 1 and 2 using a volumetric pipette to add 4.0 mL Type I Reagent Grade water to each vial.
- (3) Replace respective caps and mix thoroughly by inversion. Record dates of reconstitution and expiration on vial labels, then promptly store upright at 4-8°C. Always return calibrators promptly to refrigerator--do not leave vials at room temperature for an extended period. Store reconstituted calibrators upright at 2-8°C for up to seven days.

Calibrator Lot Validation: Each new lot of calibrators must be evaluated against the current lot prior to putting into use. (Evaluation against whole blood calibrators may be performed as needed – see Note 9). Analyze each level in duplicate within the same run over a period of two to three days (include both instruments) to verify that manufacturer-assigned values are valid. First, calibrate the run using the current lot of calibrators and analyze the controls. Analyze the new lot calibrators as unknowns immediately after the controls, running each level in duplicate. Record the values obtained including analyses from both instruments. When the tally is complete, calculate a mean to confirm the assigned bottle value or to determine new assigned values. Prior to analyzing patient specimens, verify that analysis of current lot of controls against the new lot calibrators produces results that fall within established control ranges.

New lots of calibrator may be evaluated as necessary against whole blood calibrators obtained from the NGSP CPRL at the University of Missouri (UMO calibrators). Perform this procedure when validation of new lot calibrators against current lot calibration does not confirm manufacturer-assigned values.

- (1) Obtain aliquots of UMO whole blood calibrators and UMO controls. First calibrate both instruments with UMO calibrators using their assigned values. Analyze the UMO controls, the current lot of in-house controls and both levels of new lot calibrator in duplicate as unknowns. Verify that the controls fall within their respective QC limits (preferably within 1 SD). Evaluate the results of the new lot calibrators against their manufacturer-assigned values.
- (2) Next calibrate both instruments with the new lot of Tosoh calibrators using the manufacturer-assigned values. Analyze the UMO controls and in-house controls and verify that they fall within their respective QC limits (preferably within 1 SD). If control results are acceptable, the assigned values may be used. If controls do not fall within established ranges, repeat analysis of new lot calibrators against UMO calibrators to establish new assigned values.

- (3) Additionally, the most recent set of NGSP Monthly Monitoring samples may be thawed and analyzed on both instruments and the results compared with results obtained using the current lot calibrators.

E. Preparation of Quality Control Material

Two levels of glycosylated hemoglobin control (Normal and Elevated) are analyzed in duplicate (or more) with each batch. See QC charts for controls currently in use and established ranges. Controls are prepared from whole blood drawn from a normal (Normal) and a diabetic (Elevated) individual. Stable indefinitely stored at -70°C .

Collect six 10-mL potassium-EDTA tubes from one normal or one diabetic volunteer depending on the control level to be prepared. Mix well by gentle inversion then pour blood into a 100-mL beaker containing a small magnet and place the beaker into a bucket containing wet ice. Place bucket on a magnetic stirrer set on low speed. Aliquot ~ 100 μL into 0.6 mL polypropylene microcentrifuge tubes with caps. Continue to add ice to the bucket as needed to keep beaker chilled. During preparation, aliquots may be held in an insulated bucket filled with ice until placed into boxes to be stored at -70°C (chest freezer).

At the start of each week, take one week's supply of controls from the stock supply and place in the working -70°C freezer.

Evaluate the new lot of controls according to QC/QA guidelines to establish temporary ranges prior to placing into clinical use.

College of American Pathologists (CAP) specimens and Monthly Monitoring specimens from the University of Missouri are used for proficiency testing. The TOSOH 2.2 Plus instruments are NGSP certified.

7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

The analyzer has a two-point automatic calibration function. Studies have shown the calibration to be stable for at least seven days if the system is calibrated and maintained according to the procedures provided in this guide and the G7 Automated HPLC Analyzer Operator's Manual.

Perform weekly calibration of the instrument on Mondays prior to analysis of controls and patient samples. Calibration must also be performed after repeated control failure, major maintenance or service has been performed or whenever a new column is installed.

- A. Verify that there is sufficient volume of Elution Buffers and Hemolysis & Wash Solution.
Replace if necessary.
- B. Check analyzer status.
 - If analyzer is off, press the POWER key. The analyzer begins its 10.8 minute warm-up.
 - If analyzer is in STAND-BY mode, proceed to step 3.
- C. Select CALIB from the MAIN screen. Once selected, it will be reverse highlighted. If it is necessary to change the current calibration values, select MENU, then PARAMETER. Press CALIB-1 and enter the assigned value for Calibrator 1. Press CALIB-2 and enter the assigned value for Calibrator 2.

- D. Pipette at least 800 μ L of each calibrator into sample cups. Place the sample cups in the rack with Calibrator 1 in position 1 (on the left) and Calibrator 2 next to it in position 2. Place dilutions made from the current lot of controls in positions 3 and 4. Place an empty rack on the loader to signal the end of the run.
- E. Press the START key to begin the calibration. The analyzer samples Calibrator 1 three times and Calibrator 2 two times. The analyzer discards the first measurement of Calibrator 1, and uses the remaining four measurements to calculate factors A and B. Upon completion of the calibration, the CALIB button will automatically go off. Following a successful calibration, patient and control samples will be calculated using the new factors. Record the new calibration parameters on the protocol page. Allow the instrument to analyze the controls and evaluate them before placing more specimen racks in the sample loader. If controls exceed acceptable limits, re-calibrate.

8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

A. Procedure

- (1) Turn on the autodilutor. On Mondays, prime the system with Hemolysis & Wash Solution by placing the inlet tubing into the reagent reservoir and secure the cap. (The autodilutor is stored in deionized water over the weekend.) Remove the spacers under each syringe. Set the mode switch to CONT and tap the remote switch on the probe to initiate continuous reagent dispensation. Let syringes cycle 4-5 times to purge tubing and syringes of air and to prime the system with reagent. When the syringes are on the upstroke, set mode switch back to MAN mode (manual dispense). Install the 5% spacer for the 100 μ L sample syringe and the 50% spacer for the 2.5 mL reagent syringe. For specimens with a low hematocrit, dilute the specimen using the 10% sample syringe spacer.
- (2) Daily Setup procedure:
Consult both the Daily Maintenance and the As Needed Maintenance Logs prior to starting analysis each day (see Maintenance instructions at end of procedure). Check off each required task as it is performed and initial the log.

Both analyzers are currently programmed to warm-up automatically at 7:00 AM Monday through Friday. However, if necessary to manually initiate warm-up, press the POWER key located on the operation panel to switch on the analyzer. This will initiate a 10.8-minute warm-up sequence. The analyzer Status screen displays the analyzer's current operating mode (WARMING-UP). Check for leaks and record pump pressure during the 10.8-minute WARMING-UP sequence.

To check flow rate at a time other than during the WARMING-UP sequence, first verify that the analyzer is in STAND-BY mode. Press the 'down arrow' on the MAIN screen. The valves operate as toggle switches. The valves operate as toggle switches. Press the SV-1, Sv-2 and or SV-3 valve keys appropriately until valve status line appears as follows: O X X. Press PUMP to start the pump. Wait a few seconds for flow rate to stabilize, then record. Press PUMP again to stop the pump. Press the 'down arrow' to return to the MAIN screen.

Note: The main power switch is located on the lower left side of the analyzer and must remain ON at all times. If the main power has been interrupted or switched off, the application software and default parameters must be re-loaded by inserting the System Disk in the floppy drive with the main power switch on and pressing the analyzer's POWER. Remove the current data disk from the analyzer and insert the analyzer's System disk. The analyzer automatically loads the program and begins the WARMING-UP sequence. After the LED on the drive goes out, remove the System Disk and return to a safe place. Replace the current data disk in the drive. Be sure to re-enter the current calibration parameters (and re-calibrate if necessary).

- (3) Generate a parameter printout to bracket the run by pressing MENU on the MAIN screen, then press UTILITY, then PARAM PRINT. Verify that parameters are set correctly by comparing them with the example posted inside the right door of the analyzer. Pay close attention to the current values posted for CALIB-1 and CALIB-2. Press 'EXIT' to return to the MAIN screen.
- (4) If instrument calibration is not required, run both calibrators as unknowns in positions 1 and 2, followed by dilutions made from the current lot of controls in positions 3 and 4. Place the rack in the left compartment of the sample loader. Place a second (empty) rack behind this rack. Press the START key. Allow the instrument to analyze the calibrators and controls and evaluate them before more specimen racks in the sample loader. If controls exceed acceptable limits, re-calibrate.
- (6) Protocol pages:

A day's analysis load may consist of one or more runs. It is acceptable to analyze samples in one continuous run or in several shorter runs.

 - (a) Controls: Begin with analysis of both controls in the first run. Alternate analysis of control levels in each subsequent run and at least once on each protocol page. Controls must be analyzed in duplicate (at least) each day. Evaluate against established limits.
 - (b) Patient samples: Record accession numbers and/or CIDs on the protocol page in the order in which the samples are to be analyzed.
 - (c) Calibrators (as unknowns): Analyze Calibrator 1 and Calibrator 2 as unknowns at least once per day within the batch and again to bracket all samples at the end of the day's batch. Acceptable range for calibrators analyzed as unknowns are ± 0.2 of assigned values.
 - (d) Between batch duplicate: Analyze a specimen from the previous day as a duplicate at some point in the current day's run. Result must agree within ± 0.2 of the previous value.
 - (e) Within batch duplicate: Analyze a specimen from the beginning of the run again at the end of the run. Result must agree within established duplicate range.

NOTE: The analyzer is programmed to begin a 10 minute wash mode immediately following the completion of the last sample. Once the WASH

cycle begins, you must allow it to proceed to completion (approximately 10 minutes). If washing is insufficient, column lifespan will be reduced and the result for the next sample could be affected. If there is no further input from the operation panel while the analyzer is in the STAND-BY mode, after 3 hours the analyzer will shut down automatically.

(7) Prepare samples for analysis:

Controls. Thaw aliquots and vortex briefly. Using the autodilutor, prepare hemolysates by diluting 5 uL (5% sample spacer) of each control with 1.25 mL (50% reagent spacer) Hemolysis & Wash Solution. Wipe the probe tip after drawing up sample and again after dispensing into a labeled vial. Place the sample vials in the rack.

Whole Blood. Ensure that the stopper on the tube is properly seated and that the barcode label is vertically aligned; re-affix barcode label vertically on tube if necessary. Mix each patient specimen several times by gentle inversion (or place briefly on a rotator). Then place the specimen tube in the rack in order from left to right according to its rack and position number as recorded on the protocol page. Align its barcoded label so that it faces the barcode reader (i.e., facing away from you in the rack as it's loaded on the instrument).

Note: Blood cells will begin to settle out as the tubes sit on the instrument waiting to be measured. This cell sedimentation over a period of approximately 5 hours does not affect the HbA1c result.

Low-volume samples (< 1.0 mL whole blood in tube). Using the autodilutor, prepare hemolysates by diluting 5 uL (5% sample spacer) of each low-volume patient sample with 1.25 mL Hemolysis & Wash Solution (50% reagent spacer). Wipe the probe tip after drawing up sample and again after dispensing into labeled sample vial. Place the sample vial in the rack. (Minimum dilution volume dispensed or pipetted into a sample vial is 300 uL.)

HbA1c Sample Preparation Vials. Remove caps prior to sampling! Place the Prep vial in the rack using an adapter tube.

B. Operation

- (1) Place racks in ascending order into the sample loader. Place an empty rack after the last rack to be processed. Presses START key to begin analysis. The racks will be moved automatically along the sample loader. The analyzer will prime the fluid lines with buffer for 4.4 minutes, then analyze samples at 2.2-minute intervals.

IMPORTANT: Keep tubes in the sample rack until the whole rack is processed and printed reports are available and have been reviewed

- (2) Processing automatically stops when the analyzer detects an empty rack (or 10 sequential empty spaces). When measurement ends, the analyzer will enter the WASH mode in which it washes the column by pumping buffer for 10 minutes, then enters STAND-BY mode again. Once it has started, always allow the WASH sequence to go to completion!

- (3) If there is no further input from the operation panel while the analyzer is in STAND-BY mode, after 3 hours (Off Time setting), the analyzer shuts itself off automatically.

C. Special Method Notes

- (1) To avoid an error condition during calibration, place Calibrators 1 and 2 in the first sample rack in positions 1 and 2 respectively.
- (2) Each master reagent lot number of G7 HSi Elution Buffers supplied by Tosoh is performance matched to the corresponding TSKgel G7 HSi Variant Columns. Following any announced change in supplied Tosoh TSKgel G7 HSi Variant Columns, contact Tosoh to determine suitability of existing elution buffers. Note: Hemolysis & Wash Solution may be used with any master lot of column/buffers since it is not necessary that it be matched to the other components.
- (3) The reagents must be at room temperature (15-25°C) prior to use.
- (4) Never pour or transfer reagent from one bag or container to another; results may be compromised.
- (5) When replacing the column, run at least three whole blood samples to prime the column then recalibrate the system before running quality control materials. Refer to the G7 Automated HPLC Operator's Manual for specific instructions.
- (6) Replace the filter element if pressure is greater than 9 MPa or after 400 injections.
- (7) If the column is not to be used for more than one week, remove it from the analyzer and seal the ends with the protective plugs and store in cool place at 4-15°C. Avoid direct sunlight.
- (8) At least once a day check the waste container to ensure that there is enough space remaining to accommodate a run. If necessary, empty container and add about one liter of fresh 5% sodium hypochlorite solution (household bleach).
- (9) New lots of calibrator may be evaluated as necessary against whole blood calibrators obtained from the NGSP CPRL at the University of Missouri (UMO calibrators). Perform this procedure when validation of new lot calibrators against current lot calibration does not confirm manufacturer-assigned values.
 - a. Obtain aliquots of UMO whole blood calibrators. First calibrate both instruments with UMO calibrators using their assigned values. Analyze the current lot of in-house controls and both levels of new lot calibrator in duplicate as unknowns. Verify that the controls fall within their respective QC limits (preferably within 1 SD). Evaluate the results of the new lot calibrators against their manufacturer-assigned values.
 - b. Next calibrate both instruments with the new lot of Tosoh calibrators using the manufacturer-assigned values. Analyze the UMO controls

and in-house controls and verify that they fall within their respective QC limits (preferably within 1 SD). If control results are acceptable, the assigned values may be used. If controls do not fall within established ranges, repeat analysis of new lot calibrators against UMO calibrators to establish new assigned values.

- c. Additionally, the most recent set of NGSP Monthly Monitoring samples may be thawed and analyzed on both instruments and the results compared with results obtained using the current lot calibrators.
- (10) Following major maintenance, calibrate the system. Run controls plus 5 duplicate samples. Results should agree within 0.2%. Record these results on the Rgt/Column Change Sheet found in the Rgt Change, Calibrators, etc book.

D.. Calculations

How the Analyzer Calculates Calibration Factors

$$A(\text{slope}) = \frac{(\text{Cal}_{2A} - \text{Cal}_{1A})}{(\text{Cal}_{2M} - \text{Cal}_{1M})} \quad B(\text{intercept}) = \text{Cal}_{2A} - (\text{Cal}_{2M} \times A)$$

where Cal_{1A} and Cal_{1M} are assigned and measured values for Calibrator 1, and Cal_{2A} and Cal_{2M} are assigned and measured values for Calibrator 2.

Calibration Acceptability Criteria

When the calibration procedure is completed, the analyzer automatically accepts or rejects the calibration results. If the calibration is unsuccessful, recalibration will be required. A Calibration Error message appears and the run aborts if:

- .-The two SA1c% results for Calibrator 1 differ by 0.3% or more.
- The two SA1c% results for Calibrator 2 differ by 0.3% or more.
- Any of the four calibrator results differs from its assigned value by $\pm 30\%$ or more.

E. Interpretation of Results

For detailed information about interpreting the results, see the G7 Automated HPLC Analyzer Operator's Manual.

An unusual chromatogram can result either from an abnormal sample, a procedural error, an instrument malfunction or sample-handling problem.

In general, review and question any chromatogram with the following characteristics:

1. The SA1c value is outside the normal range established by your laboratory.
 2. The SA1c peak is not detected.
 3. Total area reported is less than 500 or greater than 3000.
 4. An unidentifiable peak (i.e., P00, P01, ...) appears before the A0 peak.
 5. The retention time for the SA1c peak is less than 0.62 or greater than 0.82.
- If a result displays any of these characteristics, repeat the sample.

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If the repeated sample also displays unusual characteristics, then it is appropriate to evaluate whether the unusual result is due to an abnormal sample, a procedural error, an instrument malfunction or a sample-handling problem. For further information, see Troubleshooting in Chapter 6 of the Operator's Manual.

Important! Do not report any result that displays the characteristics above without appropriate review. The presence of any of these criteria may indicate that there is a problem or the sample may be inappropriate for measurement by this method.

Barcoded samples are scanned automatically by the analyzer and the CID number appears on the chromatographic printout in the 'SAMPLE ID' field. If a barcode is unreadable or unavailable, the rack and position numbers of the sample appear in this field instead. In such cases, always record the accession number or Lab ID on the chromatogram. Record %SA1C value from the tape onto the protocol page. Be sure to note any abnormal peak(s) (abnormal variants or POO peaks) on the protocol page.

1. Quality control evaluation:

-Calibrators (as unknowns): Acceptable range for calibrators analyzed as unknowns are ± 0.2 of assigned values.

-Controls: Values must fall within established ranges for each level.

-Batch duplicates: Results must agree within ± 0.2 of the previous batch's value.

2. Dilution studies demonstrate that the assay is linear from a Total Area of 500 to 3000. In general, review and question any chromatogram with the following characteristics:

-The SA1C value is below 4.0%. Repeat the sample to confirm. Consult a supervisor before reporting (< 4.0 %).

-Total area reported is less than 500 or greater than 3000. Repeat dilution using appropriate reagent or sample syringe spacer to obtain area results within this range.

-The SA1C peak is not detected. Repeat the sample to confirm. Do not report results. Consult a supervisor.

-An unidentifiable peak (P00, P01, ...) peak appears before the A1A or between the A1A and the A0 peaks. Do not report results. Check for clots in the sample; re-analyze. Consult a supervisor before reporting results.

NOTE: If a repeated sample also displays unusual characteristics, then it is appropriate to evaluate whether the unusual result is due to an abnormal sample, a procedural error, or sample-handling problem.

SA1C - Report % HbA1c (SA1C) to one decimal place.

F – Observe elevated HbF peak between A1B and LA1c+ peaks. Levels of fetal hemoglobin (HbF) up to 15% do not affect test results because HbF is completely resolved by the analyzer. For adult patients with HbF > 15%, send the specimen to the VA Hospital (see instructions in 'Other hemoglobin variants' section).

H-VAR –Hemoglobin variants (for example, HbS and HbC and other)

-HbS (heterozygous) – HbS appears as an H-V1 peak following the A0 peak and there is no carryover observed in the chromatograms that follow. HbS (heterozygous) does not interfere with quantitation of HbA1c. Report the HbA1c result with the following coded comment: **C7672** (*Abnormal hemoglobin variant observed*).

-HbC – HbC appears as an H-V2 peak that follows the A0 peak. There is no carryover observed in the chromatograms that follow. HbC (heterozygous) does not interfere with quantitation of HbA1c. Report the HbA1c result with the following coded comment: **C7672** (*Abnormal hemoglobin variant observed*).

-Other hemoglobin variants – may appear as a POO peak or H-VAR. Consult a supervisor. Bring the sample to the Primary Care Lab for analysis on the DCA 2000. Report the result with the comment: **HGBVC** (*Abnormal hemoglobin variant observed. HbA1c measured by the DCA 2000 immunoassay method.*)

9. REPORTABLE RANGE OF RESULTS

Elevated levels of HbA1c suggest the need for more aggressive treatment of glycemia. The American Diabetes Association recommends that a primary goal of therapy should be

HbA1c < 7%, and that physicians should reevaluate the treatment regimen in patients with HbA1c values consistently above 8%.

LINEAR RANGE (AMR): 3.0 – 19.0 %
Report results falling outside this range as <3.0 or >19.0 %.

CLINICALLY REPORTABLE RANGE (CRR): 3.0 – 19.0 %

10. QUALITY CONTROL (QC) PROCEDURES

Two levels of glyated hemoglobin control (Normal and Elevated) are analyzed in duplicate (or more) with each batch. Westgard rules are followed as outlined in the general laboratory Quality Control and Quality Assurance procedure. Controls are analyzed at the beginning of a run, periodically throughout, and at the end of a run. Controls are prepared from whole blood drawn from a normal (Normal) and a diabetic (Elevated) individual. Stable indefinitely stored at -70°C. Controls should be diluted with Hemolysis & Wash Solution to obtain a Total Area on the chromatogram in the range of 500-3000. If the value of one or more control specimens is out of the acceptable range, recalibrate the system and rerun the controls before testing patient samples.

Collect six 10-mL potassium-EDTA tubes from one normal or one diabetic volunteer depending on the control level to be prepared. Mix well by gentle inversion then pour blood into a 100-mL beaker containing a small magnet and place the beaker into a bucket containing wet ice. Place bucket on a magnetic stirrer set on low speed. Aliquot ~ 50 uL into 0.2 mL polypropylene microcentrifuge tubes with caps. Continue to add ice to the bucket as needed to keep beaker chilled. During preparation, aliquots may be held in an insulated bucket filled with ice until placed into boxes to be stored at -70°C (chest freezer). At the start of each week, take one week's supply of controls from the stock supply and place in the working -70°C freezer.

Evaluate the new lot of controls according to QC/QA guidelines to establish

ranges prior to placing into clinical use

Quality control evaluation:

Calibrators (as unknowns): Acceptable range for calibrators analyzed as unknowns are \pm 0.2 of assigned values.

Controls: Values must fall within established ranges for each level.

Batch duplicates: Results must agree within \pm 0.2 of the previous batch's value.

11. REMEDIAL ACTION IF CALIBRATION OR QC SYSTEMS FAIL TO MEET ACCEPTABLE CRITERIA

If control values are out of the acceptable range, recalibration is required. Reanalyze any patient samples after recalibration.

12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS

Dilution studies demonstrate that the assay is linear from a Total Area of 500 to 3000.

Patients who possess no hemoglobin A (i.e., homozygous variants, double heterozygotes, etc.), will not have HbA1c. Therefore, the level of glycemic control in these patients cannot be measured by this method.

Levels up to 15% Hemoglobin F (fetal hemoglobin) do not interfere with the accurate determination of the percent of HbA1c in the normal range population.

HbA1c results cannot be reported in patients with heterozygous hemoglobinopathies in which the non-A hemoglobin elutes prior to the A0 peak. In such situations, the results may be falsely increased or decreased depending upon the retention time of the abnormal hemoglobin. Peak areas of hemoglobins eluting after the A0 are not included in the calculations of Total Area on the G7 Automated HPLC System. Therefore, the presence of such hemoglobin does not interfere with the calculation of the HbA1c result. Because HbS, HbD, and HbC elute after the A0, glycemic control for heterozygous patients with Hemoglobin AS, AD and AC may be accurately monitored using HbA1c on the G7 Automated HPLC System when the Variant Analysis Mode is used.

Various substances other than sugars can form adducts with hemoglobin, thereby altering its charge characteristics. Falsely elevated results can occur if these adducts co-migrate with the stable HbA1c. Literature cites examples including individuals with opiate addiction², lead poisoning, uremia and alcoholism³, as well as those receiving large amounts of aspirin (acetylated hemoglobin)⁴. Clinically, the most important of these possible interfering adducts occurs in uremia³. The interference correlates with the BUN (blood urea nitrogen) and appears to result at least in part from the carbamylation of hemoglobin by urea-derived cyanate (carbamyated hemoglobin).⁵ Studies on the G7 Automated HPLC in the Variant Analysis Mode indicate that the carbamyated and acetylated hemoglobin derivatives are eluted in the labile fraction. No interference in stable HbA1c results from these derivatives has been observed up to concentrations of 5.5% of carbamyated hemoglobin and 2.7% of acetylated hemoglobin.

The results obtained from this assay should be used in conjunction with other data (for example, signs and symptoms, duration of diabetes, results of other tests, age of patient, clinical impressions, degree of adherence to therapy, etc.). This test should not be used to diagnose diabetes mellitus. The performance characteristics for diagnosis have not yet been established.

Abnormal Red Cell Survival

The life span of red blood cells is shortened in patients with hemolytic anemias, depending upon the severity of the anemia. As a consequence, specimens from such patients will exhibit decreased glycohemoglobin levels compared to patients with expected red cell life span.

The life span of red blood cells is lengthened in polycythemia or post-splenectomy patients. Specimens from such patients may exhibit increased glycohemoglobin levels. For these types of patients, reference range values will not apply.

13. REFERENCE RANGES (NORMAL VALUES)

REFERENCE RANGE: 4.3 – 6.0 % (DCCT/EDIC normal range)

14. CRITICAL CALL RESULTS (“PANIC VALUES”)

Early Reporting Results for NHANES:

Notify the NHANES Medical Officer of any SA1c% results greater than 6.9%. The contact person will report these results as soon as possible.

15. SPECIMEN STORAGE AND HANDLING DURING TESTING

Any specimens not analyzed on the day of arrival in the laboratory are stored in the refrigerator (4°C - 8°C). Upon completion of analysis, specimens are stored for 1 week. NHANES specimens are frozen at -70°C and discarded after 1 year.

16. ALTERNATIVE METHODS FOR PERFORMING TEST OR STORING SPECIMENS IF TEST SYSTEM FAILS

The laboratory has 2 instruments for performing glycohemoglobins. If neither instrument is available for use, the specimens are stored at 4°C until testing can be performed.

Unopened and stored at 2-8°C, the HbA1c Calibrator and Control Sets are stable until the expiration date printed on the label. After reconstitution, calibrators are stable for one week when stored at 2–8°C. Reconstituted Controls are stable for five (5) days when stored at 2–8°C.

Unopened Elution Buffers HSi Variant (S) 1, 2, and 3 are stable until the expiration date printed on the label. After opening, Elution Buffers (S) is stable for three months. Store at 4-25°C.

Unopened Hemolysis & Wash Solution is stable until the expiration date printed on the label. After opening, Hemolysis & Wash Solution is stable for three months. Store at 4–25°C.

The unopened TSKgel G7 HSi Variant column is stable indefinitely when stored at 4–15°C away from direct sunlight. Columns are warranted for 1500 injections. If columns are performing well based on acceptable quality control results and chromatogram quality, they may be used past 1500 injections until they no longer meet performance criteria.

17. TEST RESULT REPORTING SYSTEM; PROTOCOL FOR REPORTING CRITICAL CALLS (IF APPLICABLE)

NHANES SA1c% results are entered onto a spreadsheet provided electronically by WESTAT, Inc for NHANES.

To access the spreadsheet click on My Computer → Z drive → User → Dep Labs → Collab Studies → NHANES → Glyhb 004.

Choose the file named with the corresponding box number.

Enter the analysis date, run number, technologist's initials, SA1c%, and result comment code.

The spreadsheet will be sent electronically by the contact person.

Early Reporting Results for NHANES:

Notify the NHANES contact person of any SA1c% results greater than 6.9%. The contact person will report these results as soon as possible.

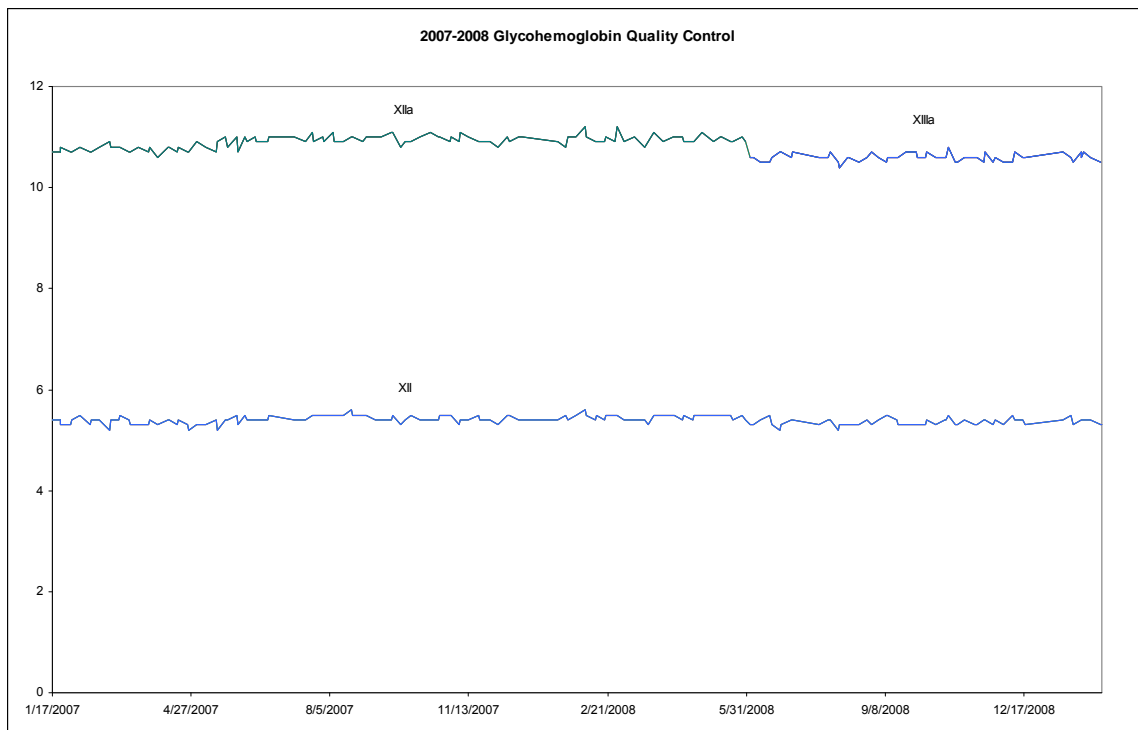
18. TRANSFER OR REFERRAL OF SPECIMENS; PROCEDURES FOR SPECIMEN ACCOUNTABILITY AND TRACKING

All shipments are recorded on the NHANES Shipping Log upon receipt. Actions taken during the course of analysis, result reporting, and specimen retention are also recorded on the log.

19. SUMMARY STATISTICS AND QC GRAPHS

Summary Statistics for Glycohemoglobin by Lot

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
XII	111	1/17/2007	5/30/2008	5.422	0.081	1.5
XIIa	111	1/17/2007	5/30/2008	10.905	0.122	1.1
XIII	56	6/3/2008	2/11/2009	5.357	0.074	1.4
XIIIa	56	6/3/2008	2/11/2009	10.598	0.080	0.8



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