

Laboratory Procedure Manual

Analyte: **Glycohemoglobin**

Matrix: **Whole Blood**

Method **Tosoh A1c 2.2 Plus
Glycohemoglobin Analyzer**

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

Contact: Dr. Michael Steffes

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

Hemoglobin subfractions formed by the glycation of the alpha or beta chains of hemoglobin A1 (HbA) are collectively known as glycosylated or glycated hemoglobins. Hemoglobin A1c, the best-defined of these, is formed by the reversible condensation of the carbonyl group of glucose and the amino group at the N-terminus of the beta chain of hemoglobin A, resulting in a labile aldimine or Schiff base. As the red cell circulates, some of the aldimine undergoes a slow, irreversible conversion (Amadori rearrangement) to a stable ketoamine form (HbA1c). As blood glucose levels rise, the increase in glycated hemoglobin is proportional to both the level of glucose and the lifespan of the red cell. Hemoglobin A1c measurements are used in the clinical management of diabetes to assess the long-term efficacy of diabetic control. The glycated hemoglobin result is a reflection of the mean daily blood glucose concentration and the degree of carbohydrate imbalance over the preceding two to three months.

In the past, accurate measurement of stable HbA1c was possible only after removing labile HbA1c by pretreatment. In this assay, the stable (SA1c) and labile (LA1c) forms can be individually resolved on the chromatogram without manual pretreatment, allowing accurate measurement of the stable form of HbA1c. The analyzer dilutes the whole blood specimen with Hemolysis & Wash Solution, and then injects a small volume of the treated specimen onto the HPLC analytical 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 (A1c, A1b, F, LA1c, SA1c, A0 and H-Var) are subsequently removed from the column material by step-wise elution using Elution Buffers 1, 2 and 3, each with a differing salt concentration. The separated hemoglobin components pass through the 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. Analysis requires three minutes.

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

A. Instrumentation

- (1) A1c 2.2 Plus Glycohemoglobin Analyzer. Part # 018379, with 90 sample loader, Par # 018442. Tosoh Medics, Inc., 347 Oyster Pt. Blvd., Suite 201, So. San Francisco, Ca 94080.

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- (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 # 018577, 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 # 018550, 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 2.2 Plus, 10 roll/box. Part # 018417. Tosoh Medics, Inc.
- (4) Sustaining tube, Dilution Sample, 10 mL, 50/pkg, Part # 017093. Tosoh Medics, Inc.
- (5) Adapter Ring, Dilution Sample, 5/pkg, Part # 017094. Tosoh Medics, Inc.
- (6) Adapter Ring, Sample Rack: 12 mm, Part # 018496; 13 mm, Part # 018433; and 14 mm, Part # 018497.
- (7) Sample vials, 1.8 mL (no caps), 1000/pkg. Part # 007419. 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:
- (10) 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.
- (11) 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.
- (12) 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.

(13) Labels, 4 sheets of 25 blank labels each.

(14) Instruction Manual.

C. Reagent Preparation

- (1) Elution Buffer HSi Variant No. 1, (S) Part # 018572 (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 # 018573 (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 # 018574 (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) Hemolysis & Wash Solution, (S) Part # 018430 (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.

Reconstitute Calibrators 1 and 2 by adding 4 mL Milli-Q water to each vial then mix gently 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.

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 1). Analyze each level in

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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 glycohemoglobin 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

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. The analyzer utilizes a two-point automatic calibration function. Select SET from the Status screen. Press DAILY CALIB so that it appears reverse-highlighted. The STATUS screen will display 'CALIB. : YES'. Check the parameter printout posted inside the analyzer door to be sure the current calibrator values are entered.

If necessary to change the current calibration values, press SET on the Status screen, then CALIB-1. An asterisk (*) will appear next to the parameter to be changed. Input the assigned value for Calibrator 1. Your entry will appear along the bottom of the screen; press the 'left-hand arrow' key on the screen to enter this value. Then press CALIB-2 and enter the assigned value for Calibrator 2 in the same manner. Press the 'up' arrow to return to the Status screen.

Pipette 950 uL (minimum volume 800 uL) of each calibrator into each of two sample vials. Place Calibrator 1 in position 1 (on the left) and Calibrator 2 next to it in position 2 in the rack using adapter rings and sustaining tubes. Place dilutions made from the current lot of controls in positions 3 and 4. Place the rack into the left compartment of the sample loader. Then place a second (empty) rack behind this rack. (The analyzer senses the end of a run after it detects 10 sequential empty positions.) Press the START key to begin the calibration. The analyzer measures Calibrator 1 three times and Calibrator 2 two times. The analyzer discards the first measurement and uses the remaining four measurements to calculate factors A (slope) and B (intercept). Record the new calibration parameters on the protocol page. Allow the instrument to analyze the controls (see step 7. 'Prepare samples for analysis') and evaluate them before placing more specimen racks in the sample loader. If controls exceed acceptable limits, re-calibrate.

How the Analyzer Calculates Calibration Factors

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

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

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.

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 µL 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 (located at lower left under the screen) to switch on the analyzer. This will initiate an 18-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 18-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 MENU then MAINT on the Status screen. The valves operate as toggle switches.

Press the SV-1, Sv-2 and or SV-3 valve keys appropriately until valve/degasser status line appears as follows: 1/2/3/D OP/CL/CL/OP. Wait a few seconds for flow rate to stabilize, then record. Press the 'up arrow' at lower right of the screen display to return to the Status 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 key on the bottom of the operation panel. 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) Prime the system daily by running five whole blood specimens prior to calibration. First, turn off calibration by selecting SET on the Status screen then press DAILY CALIB so that it is no longer reverse-highlighted. Return to the Status screen by pressing the 'up arrow' key. Press the START key (located on the operation panel under the screen) so that once the WARMING-UP sequence is complete, the analyzer will immediately begin sampling the priming specimens. Once analysis of the priming specimens is complete, use the paper feed button (located behind the screen) to advance the paper to reveal the entire chromatogram.
- (4) Generate a parameter printout to bracket the run by pressing MENU on the Status screen, then press UTILITY, then PARAM PRINT. Verify that parameters are set correctly by comparing them with the example posted inside the left door of the analyzer. Pay close attention to the current values posted for CALIB-1 and CALIB-2. Press the 'up arrow' to return to the Status screen.

After equilibration, the analyzer will enter the STAND-BY mode unless it has been programmed to advance to analysis of the priming specimens as directed in the previous step. In this instance, after analysis of the priming specimens is complete, the analyzer will enter the 'delayed' WASH mode.

- (5) 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 acceptable to analyze samples in one continuous run or in several shorter runs. The 3-character Batch ID ('NO.') appears in upper left corner of chromatographic printout. A new batch is initiated after the daily WARMING-UP sequence is completed or any time the POWER button on the Operation panel is turned off then on again (such as after a power failure).

 - (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) 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 permit an 84-minute lag time between entering 'delayed' WASH mode and going into 'active' WASH mode (see Step 9). During this 84-minute period, you may initiate a new batch of analyses by pressing START. However, once the 'active' WASH cycle begins, you must allow it to proceed to completion (approximately 15 minutes). If washing is insufficient, column lifespan will be reduced and the result for the next sample could be affected. After the 'active' WASH cycle is completed, re-run controls prior to analyzing samples.

The 84-minute lag period can be circumvented by pressing the STOP button (located on the operation panel) ONCE before this time period has elapsed. This will place the analyzer in STAND-BY mode. 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 will shut down automatically.

(7) Prepare samples for analysis:

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 μ L (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 using an adapter ring and sustaining tube. (Minimum dilution volume dispensed or pipetted into a sample vial is 300 μ L.) **WARNING:** The sustaining tube must always be used with an adapter ring. First place the adapter ring into the sustaining tube, then put the sample vial into the ring; otherwise a rack position error will occur.

HbA1c Sample Preparation Vials. Remove caps prior to sampling! Place the Prep vial in the rack using an adapter ring and sustaining tube. **WARNING:** The sustaining tube must always be used with an adapter ring. First place the adapter ring into the sustaining tube, then put the prep vial into the ring, otherwise a rack position error will occur.

B. Quality Control Materials

Controls: Thaw aliquots and vortex briefly. Using the autodilutor, prepare hemolysates by diluting 5 μ L (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 using adapter rings and sustaining tubes. **WARNING:** The sustaining tube must always be used with an adapter ring. First place the adapter ring into the sustaining tube, then put the sample vial into the ring; otherwise a rack position error will occur. Adapter rings limit the maximum vertical movement of the sampling needle. Sampling needle may bend or break as it pierces bottom of vial if an adapter ring is not used.

C. Operation

- (1) Place racks in ascending order into the left compartment of the sample loader with the first rack nearest you (this rack will advance first to the sampling station). Place an empty rack after the last rack to be processed. Press 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, and then analyze samples at 3-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 'delayed' WASH mode. Tosoh parameters have been modified so that the end of run wash is delayed for **84 minutes**. This allows the operator to program and analyze samples out of sequence ('stat' or urgent samples) or perform additional runs after the current run has ended. If no further runs are performed within 84 minutes from entering 'delayed' WASH mode, the analyzer will enter the 'active' WASH mode in which it washes the column by pumping buffer for 15 minutes, then enters STAND-BY mode again. Once it has started, always allow the 'active' 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.

D. Special Method Notes

- (1) 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.
- (2) Dilution studies demonstrate that the assay is linear from a Total Area of 800 to 8000. In general, review and question any chromatogram with the following characteristics:
 - (a) The SA1C value is below 3.4%. Repeat the sample to confirm. Consult a supervisor before reporting (< 3.4 %).
 - (b) Total area reported is less than 800 or greater than 8000. Repeat dilution using appropriate reagent or sample syringe spacer to obtain area results within this range. c.

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

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.

- (3) **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 22% do not affect test results because HbF is

completely resolved by the analyzer. For adult patients with HbF > 22.0%, send to the V.A. Report the V.A. result with the comment C7671.

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

- HbS (heterozygous) – HbS appears as an H-VAR 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 also appears as an H-VAR peak that follows the A0 peak however carryover is observed in one or more chromatograms that follow. Carefully examine the next 8-10 chromatograms! Observation of residual variant hemoglobin may be delayed for as many as 4-5 samples.

IMPORTANT: You must repeat all specimens that follow this type of H-VAR chromatogram up to and including the samples that exhibit the carryover. This may require repeating as many as 8-10 sequential specimens. Consult a supervisor. The SA1C result is reportable in the presence of HbC (look for the classic chromatographic pattern).

- Other hemoglobin variants – may appear as a POO peak or H-VAR. Consult a supervisor. Send the POO/H-VAR sample to the Minneapolis VAMC for analysis (see below).

Samples exhibiting an abnormal peak(s) inconsistent with heterozygous HbF or HbS must be sent to Minneapolis VAMC for analysis by boronate affinity method. To send samples to VAMC, aliquot ~1 mL whole blood into a labeled NUNC vial and place in a thermos filled with wet ice. Send thermos via courier to:

Metabolic Lab 111G
VA Medical Center,
Minneapolis, MN 55417
Ext 4424

When the VAMC report comes back, tape one copy on the back of the GLYHB protocol page and place the other in the folder at the desk. Report results appending the coded comment, **C7671** (*Abnormal hemoglobin variant observed not Hgb S. Red cell survival in these conditions can be shortened; therefore it may not be valid to compare this result with normal reference ranges for Hgb A1c. This result equals the percent Total Glycated Hemoglobin as assayed by the BioRad Variant II boronate affinity method (normal range 3.9 to 6.3%), in use as of 2/21/02, at the Minneapolis Veterans Administration Medical Center, Minneapolis, MN 55417*).

9. REPORTABLE RANGE OF RESULTS

REPORTABLE RANGE: 3.4 – 18.8 % (manufacturer's insert)
Report results falling outside this range as <3.4 or >18.8 %.

10. QUALITY CONTROL (QC) PROCEDURES

Two levels of control are assayed each time the glycohemoglobin method is performed. 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.

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.

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

Icterus (as indicated by free and conjugated bilirubin concentrations up to 200 mg/dL), lipemia (as indicated by triglyceride concentrations up to 3600 mg/dL), and hemoglobin (concentrations up to 4500 mg/dL) do not interfere with the assay.

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.

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

Glycohemoglobin in Whole Blood using Tosoh 2.2 Plus
NHANES 2007-2008

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.

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

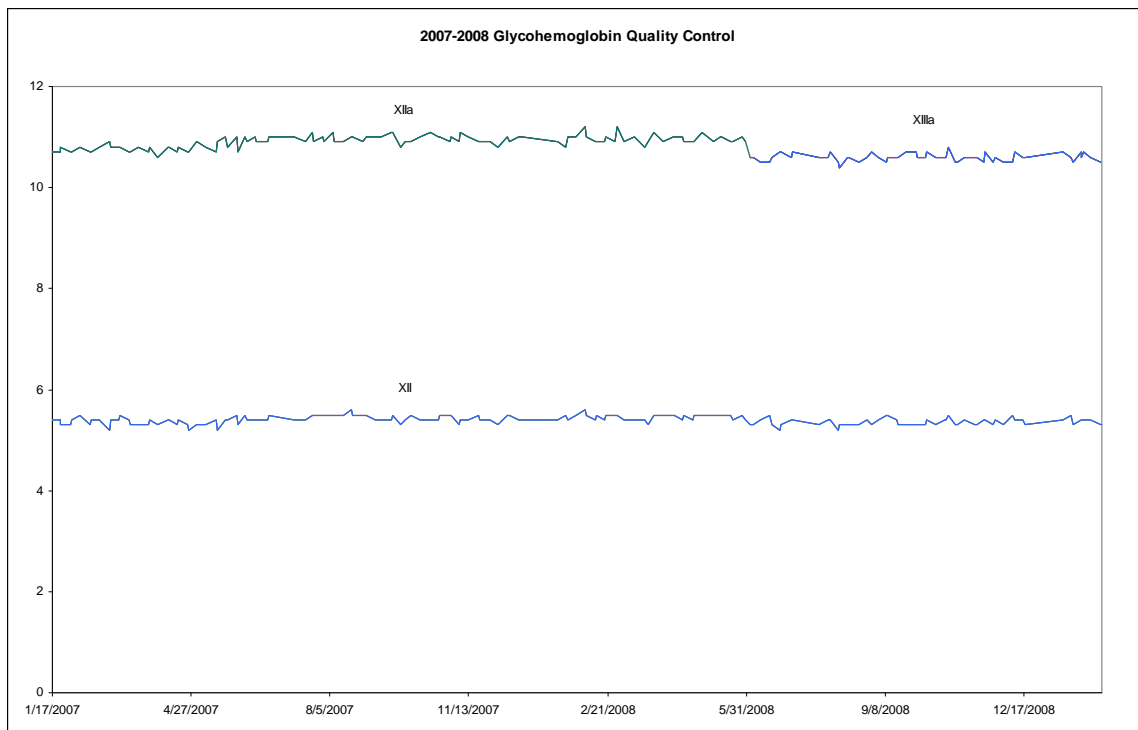
All shipments are recorded on the NHANES Shipping Log upon receipt. Actions taken during the course of analysis, result reporting, and specimen retention are also recorded on the log.

Glycohemoglobin in Whole Blood using Tosoh 2.2 Plus
NHANES 2007-2008

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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