Laboratory Procedure Manual

Analyte: **Glycohemoglobin**

Matrix: **Whole Blood**

Method Tosoh **G8 Glycohemoglobin Analyzer**

as performed by: University of Minnesota at Columbia
Columbia, Missouri

Contact: Dr. Randie Little

Important Information for Users

The University of Columbia 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_G	LBXGH	Glycohemoglobin (%)

The method changed within 2011-2012 from Tosoh G7 Automated HPLC Analyzer to the Tosoh Automated Analyzer HLC-723G8. There will be two methods associated with this data.

1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

Hemoglobin A1c measurements are used in the clinical management of diabetes to assess the long-term efficacy of diabetic control. 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:

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).
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).

The Tosoh Automated Glycohemoglobin Analyzer HLC-723G8 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 (3 uL) with Hemolysis & Wash Solution, and then injects a small volume of this specimen onto the TSKgel G8 Variant HSi 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, HV2) are subsequently removed from the column by performing a step-wise elution using the varied salt concentrations in the Variant Elution Buffers HSi 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 Tosoh Automated Glycohemoglobin Analyzer HLC-723G8 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 or slow 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 (chap 1).

The Tosoh G8 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).

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 unto 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 flouride are acceptable. Whole blood specimens are stable up to fourteen days stored at 2-8^{o}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^{o}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) G8 Automated HPLC Analyzer. Part # 019327, with 90 sample loader, Par # 018442. 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 1500 counts; replace as necessary (as indicated by appearance of chromatograms). Stable indefinitely when stored at 4-15^{o}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 9 Mpa.

(3) Thermal paper for G7, 10 roll/box. Part # 019563. Tosoh Medics, Inc.

(4) 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^{o}C. Includes supplies sufficient for 100 test samples:

(5) 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^{o}C.

(6) 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.

(7) 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.

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

(9) Instruction Manual.

C. Reagent Preparation

018767	Tosoh Hemoglobin A1c Calibrator Set	
	Calibrator 1 (approximately 5.5%)	5 x 4 mL
	Calibrator 2 (approximately 10.5%)	5 x 4 mL
	Buffered human red blood cells, 2 mg/mL Human hemoglobin	
220232	Tosoh Hemoglobin A1c Control	
	Normal	4 x 0.25 Ml
	Abnormal	4 x 0.25 Ml

Unopened and stored at 2-8°C, the Tosoh Hemoglobin A1c 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. Refer to the control package insert for stability data.

021955 TSKgel G8 Variant HSi 1 each
The unopened TSKgel G8 Variant HSi column should be stored at 4-15°C in a cool location away from direct sunlight. The column is stable until the expiration date printed on the label. Columns are warranted for 2500 injections. ARDL has shown that column may last up to 3500 injections or more.

021956 G8 Variant Elution Buffer HSi No.1 (S) 1 x 800 mL
021957 G8 Variant Elution Buffer HSi No.2 (S) 1 x 800 mL
021958 G8 Variant Elution Buffer HSi No.3 (S) 1 x 800 mL
Unopened G8 Variant Elution Buffers HSi (S) 1, 2, and 3 are stable until the expiration date printed on the label. After opening, Elution Buffers are stable for three months. Store at 4-30°C.

018431US HSi Hemolysis & Wash Solution (L) 1 x 2000 mL
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 4-30°C.

021600	Filter Element	5/pkg
018581	Sample Cups	1000/pkg
019563	Thermal Paper 10 rolls/pkg	
018723	Supply Line Filters for Buffer Lines	1/pkg
019500	Sampling Needle Assembly	1 each

Note: Volumetric pipettes are required but not supplied by Tosoh Bioscience, Inc.

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^{o}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^{o}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 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

ARDL in-house controls. 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^{o}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^{o}C freezer.

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

Biorad Lyphocheck Hemoglobin A1C Linearity Chek Set #120 (Human whole blood based control 4 x 0.5 mL, 1 of each level) is used.

Unopened and stored at 2-8°C, the Tosoh Hemoglobin A1c linearity sets are stable until the expiration date printed on the label. After reconstitution, sets are stable for one week when stored at 2-8°C. Refer to the control package insert for stability data.

F. Results

- If the SA1c peak is not detected, review and question chromatogram.
- The SA1c measuring range is 3.1 – 19.0%.
- The ideal retention time for SA1c is 0.59 minutes (acceptable range 0.57-0.61).
- Results will not be reported if the Total Area (TA) is less than 500 which can be seen in severe anemia. Results will not be reported if the TA is greater than 4000 which can be seen in polycythemia. (See "abnormal red cell survival in previous section). The optimal goal for Total Area is between 700-3000. However a TA in the range of 500-4000 is acceptable and reportable for whole blood specimens.
- The chromatogram must be examined for any unidentifiable peaks (i.e., P00, P01,) before the A0 peak. Do not report the result if these peaks exist.
- When there is a question concerning the chromatography, repeat the sample. If the repeated sample also displays unusual characteristics, 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. **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. Consult ARDL binder with abnormal chromatograms for examples of various abnormalities.
- Controls must be in the acceptable range.
- Check results for error flags and take appropriate action.

- For further information, see the Troubleshooting Section in the Tosoh Automated Glycohemoglobin Analyzer HLC-723G8 operating manual.

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.

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 10% do not affect test results because HbF is completely resolved by the analyzer.

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

- HbS (heterzygous) – 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** (A*bnormal 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** (A*bnormal hemoglobin variant observed).*

- Other hemoglobin variants – may appear as a POO peak or H-VAR. Consult a supervisor. Bring the sample to Primary Care Clinic for analysis on the DCA 2000 instrument. Report the result with the comment: **HGBVC** (A*bnormal hemoglobin variant observed. HbA1c measured by the DCA immunoassay method.)* This policy DOES NOT apply to study participant resulting since continuity of methodology is paramount. This policy only applies to clinical resulting. Study participants would be resulted as (*;NA-USDET).* This would report as NA for result, with the comment unsatisfactory determination.

G. Dilutions

For low volume whole blood samples preparation involves an autodilutor and diluting whole blood with Hemolysis & Wash Solution. Install the 5% spacer for the 100 uL sample syringe and the 40% spacer for the 2.5 mL reagent syringe (1:200 dilution). This same dilution is used for samples and controls. For specimens with a low hematocrit, dilute the specimen

using the 10% sample syringe spacer. (1:125 dilution). Dilute the specimen so that the Total Area reported is within the range of 500-4000.

The recommended dilution for packed red cells is 1:200. Continue to use the 5% spacer for the 100 uL sample syringe and the **60%** spacer for the 2.5 mL reagent syringe(1:300 dilution). Dilute sample so that the total area is 1000-2000 for Variant Analysis Mode.
The exact dilution is not critical. For detailed instructions regarding specimen dilutions, refer to the G8 Variant Analysis Mode Operator's Manual.

H. Maintenance

DAILY SETUP PROCEDURE

1. **Check waste container.** If necessary, empty and add 5% bleach.

2. **Both analyzers are currently pre-programmed to warm-up at 7:00 AM Monday through Friday.** If necessary to manually initiate warm-up, press the POWER key located on the operation panel to switch on the analyzer. (See information below on manual start up**).

3. **Check for leaks.** During warm-up, check for leaks, especially at filter and column connections. If a leak is found, tighten connections.

4. **Verify pressure is +/- 4 MPa** of value indicated on the column inspection report **and record.** Press the Down‟ arrow to display the second Main screen. Verify that pressure stabilizes column value +/**-.** Record pressure in maintenance log. If pressure is greater than 4 MPa of assigned value, replace filter element. If pressure is less than 4MPa of assigned value, check for leaks and tighten fittings.

   - Low pressure during pumping may indicate air in the system. Check the lines connecting the buffers to the analyzer. If air is observed in the line, see procedure for removing air from buffer lines in operator's manual. If the pressure does not rise during pumping, air may be present on the outlet side of the pump (see procedure for removing air from the pump outlet in operators manual).

5. **Record the instrument temperature.** The temperature should be 25.0°C ± 1.0°C.

6. **Check F/C (filter and column) counts.**
   Replace filter element at or before 400 injections (see procedure for replacing the filter element. Replace column as indicated by appearance of chromatograms. Column is guaranteed for 2500 counts; replace as necessary (ARDL routinely utilizes column to 3000-3500 if chromatograms and retention times are acceptable).

7. **Check Smart Media card space. Format a card if necessary before starting**.(See operator's manual for formatting instructions.) Press MENU on the MAIN screen then CARD to check remaining disk storage space. The CARD screen shows how full the CARD is in the upper left corner. A single CARD holds up to 12,000 sets of assay results (including chromatograms). Return to Main screen, which should display AUTO SAVE: YES.

8. **Check buffers and hemolysis-wash solution for sufficient volume.** (See procedure for installing buffers.)

9. **Check printer paper.** A single roll is enough for ~350 results. (See procedure for installing printer paper in operator's manual.)

10. **Print the parameters.** From the MAIN screen select MENU. Press UTILITY and PARAM PRINT.

Reagent and Column Preparation and Installation:

Installing G8 Variant Elution Buffer HSi

1. Press the **STOP** key to put system in STAND-BY status.
2. Remove buffer containers to be replaced.
3. Break the seal on the storage caps of the new buffer containers, leaving the caps in place.
4. Carefully place the capped buffer bags on the rack on the instrument. Verify that the buffer bag is supported on the rack by the octagonal shaped lip at the base of the threads.
5. For each reagent, remove the cap, carefully squeeze the buffer bag by hand to minimize air pockets, and then place the appropriate color-coded tubing into the corresponding bag. Ensure that the end of the tubing is touching the bottom of the container.
6. Securely tighten reagent caps.
7. From the MAIN screen, select **MAINTE**, then **REAGENT CHANGE**. Once the buffers have been correctly installed, select the appropriate buffer, and then press **CHANGE**.
8. Record reagent lot and date in use on daily protocol page. If lot number changes, record on reagent/column lot change sheet in G8 Documentation binder.

Installing Hemolysis & Wash Solution

1. Press the **STOP** key to put the system in STAND-BY status.
2. Remove the empty container.
3. Break the seal on the storage cap of the new container, leaving the cap in place.
4. Place the Hemolysis & Wash Solution bottle on the bench at the left side of the instrument, beside the H/W Solution port.

5. Remove the cap, then place the Hemolysis & Wash Solution tubing into the container. Ensure that the end of the tubing is touching the bottom of the container.
6. Securely tighten reagent cap.
7. From the MAIN screen, select **MAINTE**, then **REAGENT CHANGE**. Once the Hemolysis & Wash Solution has been correctly installed, press **H/W**, then press **CHANGE**.
8. Record reagent lot and date in use on daily protocol page. If lot number changes, record on reagent/column lot change sheet in G8 Documentation binder.

Installing TSKgel G8 Column

1. Press the **STOP** key to put the system in STAND-BY status.
2. Unscrew column fittings and remove used column.
3. Remove protective plugs from new column. Do not discard the plugs, as they are needed for storage.
4. Verify that the column master lot matches the Elution buffer lot.
5. Check flow direction - the arrow on the column should point to the left as it is placed on the instrument.
6 . Slide the inlet tubing until it extends ¼ inch past each end fitting.
7. Connect the tubing to the inlet (right) side of the column. Take special care that buffer coming from the tube does not spill onto the analyzer unit by holding absorbent paper or gauze at the outlet end of the column when priming.
8. From the second page of the MAIN screen, press **PUMP FLOW** to start pumping the buffer. When buffer solution flows from the outlet (left) side of the column, press **PUMP FLOW** again to stop the pump.
9. Connect the tubing to the outlet side of the column.
10. Press **PUMP FLOW** to start pumping and check for leaks. The pressure should rise to the pressure level that is indicated on the column inspection report + 4 MPa. If leaks occur, tighten fittings.
11. When the pressure reaches a steady state, press **PUMP FLOW** again to stop pumping.
12. From the MAIN screen, select **MAINTE**, then **REAGENT CHANGE**. Press **COLUMN RESET** to set column count back to zero.
13. Before calibrating the newly installed column, analyze at least three whole blood samples to prime the column. Calibrate the system and 5 duplicate specimens and controls.
14. Record column lot and date in use on daily protocol page. If lot number changes, record on reagent/column lot change sheet in G8 Documentation binder. Also, record column change on daily maintenance chart.

Replacing the Filter Element

1. Verify that analyzer is in STAND-BY mode.
2. From the MAIN screen, press the „Down‟ arrow.
3. Open valve 1. Press SV1 to open valve 1. Close valves 2 and 3 if they are open. Valve status appears OX X.
4. Open the door below the display.

5. Remove the brown filter outlet (peek) tubing from the top of the filter assembly.
6. Loosen the top of the filter holder assembly by turning it counterclockwise. `Remove the filter holder by pulling it straight up.
   Lightly press the top of the holder to remove the old filter element. If salt crystals are present in the holder rinse with distilled or deionized water to clean. Position the new element paying attention to how it is oriented. The white colored surface should be visible.
7. Firmly tighten the top of the filter holder assembly by hand until no further tightening is possible.
8. Slide outlet tubing until it extends ¼ inch past the end of the tubing. Connect the outlet side tubing.
9. Press the PUMP key to start Elution Buffer delivery. Confirm that the pressure reaches 6 MPa or more with no leaks from the filter housing or tubing connections. If a leak is found, tighten the assembly further. 11. Record filter change on daily maintenance chart.

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 G8 Automated HPLC Analyzer Operator's Manual.

Be sure to calibrate in the following situations. The analyzer should also be calibrated when changing any assay conditions.

Once per week (Monday) or if drift in QC is noted
The Tosoh G8 Automated HPLC Analyzer is a very precise instrument and QC remains the same day after day. If there is a drift in QC values, it is necessary to recalibrate.

When control values assayed are out of range
Calibrate when the control assay value falls outside the standard range. Measure the control sample again to confirm that it falls within the QC range before assaying a patient sample.

After column replacement
Analyze three whole blood specimens to prime the column. Then calibrate, followed by controls and 5 duplicate specimens. Controls must be in acceptable range and dups within +/- 0.2% of previous.

After analyzer maintenance or service
Calibrate after a periodic maintenance has been performed. Also analyze controls and 5 duplicate specimens.

When set assay conditions of the analyzer are changed
Calibrate when a set parameter value of the analyzer such as the flow factor is changed.

Calibrator Preparation

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 4-8oC. Always return calibrators promptly to refrigerator--do not leave vials at room temperature for an extended period.
4. Store reconstituted calibrators upright at 2-8°C for up to seven days.

To start calibrations:

1. Verify that there is sufficient volume of Elution Buffers and Hemolysis & Wash Solution.
   Replace if necessary.
2. Check analyzer status.
   • If analyzer is off, press the POWER key. The analyzer begins its 9.0 minute warm-up.
   • If analyzer is in STAND-BY mode, proceed to step 3.

3. 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.

4. Pipette at least 400µL 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.

5. 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.

Calibration Acceptability Criteria

The No. 1 and No. 2 samples on the first sample rack are treated as CALIB-1 and CALIB-2.
CALIB-1 is the low value calibrator (approximately 5.5%) and CALIB-2 is the high value calibrator (approximately 10.5%). The low value calibrator is assayed 3 times and the high value calibrator is assayed 2 times for a total of 5 times.
The first assay result for CALIB-1 is discarded and the average HbA1c% of the 2nd and 3rd assay is calculated as the result for CALIB-1. The average HbA1c% of the 4th and 5th assay is calculated as the result for CALIB-2. Based on the

assay results and the assigned values, the following linear equation is used to calculate the calibration factors:

Object of correction: HbA1c%
Correction formula: (HbA1c% after correction) = A x (HbA1c% before correction) + B
A = (CALIB-2 assigned value - CALIB-1 assigned value) / (CALIB-2 assayed value
CALIB-1 assayed value)
B = CALIB-2 assigned value - (CALIB-2 assayed value x A)

The calculated calibration factors are automatically input in the PARAMETER screen and displayed on the main screen along with the calibration date in the form: Y = AX+B.

When the calibration procedure is completed, the analyzer automatically accepts or rejects the calibration results. If the calibration is unsuccessful, recalibration will be required. When an error occurs, the assay automatically stops, and after washing, the analyzer enters the STAND-BY state. Samples placed behind the calibrator will not be assayed. When the operation is again started, calibration is performed again because it has not been completed ,

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 ±30% or more.

Calibration errors could be caused by the following:
1. The calibrator has been left for more than 1 week after dilution or has been left at room temperature for a long period of time.
2. The filter or column is clogged and the pressure is high.
3. There is a leak.
4. Samples other than the calibrator were assayed.

Perform calibration again after correcting any of the above conditions.

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 uL sample syringe and the 40% spacer for the 2.5 mL reagent syringe.

****Both analyzers currently programmed to warm-up automatically at 7:00 AM Monday through Friday.** 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 9.0 minute warm-up sequence. The analyzer MAIN screen displays the analyzer's current operating mode (WARMING-UP). Check for leaks and record pump pressure during the 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. Press the SV1, SV2 and or SV3 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 left rear 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** smart media card, turning on the main power switch, and pressing the analyzer's POWER key. Remove the current smart media data card (32-128 MB memory for storing analysis data) from the analyzer and insert the analyzer's **system** smart media card. The analyzer automatically loads the program and begins the WARMING-UP sequence. After the LED on the drive goes out, remove the **system** card and return to a safe place. Replace the current smart media data card in the drive. Be sure to re-enter the current calibration parameters (and re-calibrate if necessary).

(2) Consult both the Daily Maintenance and the As Needed Maintenance Logs prior to starting analysis each day. Check off each required task as it is performed and initial the log.

(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, analyze 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 placing more specimen racks in the sample loader. If controls exceed acceptable limits, re-calibrate.

(5) 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.

- 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.

- Patient samples: Record accession numbers and/or CIDs on the protocol page in the order in which the samples are to be analyzed.

- 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.

- 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.

- Within batch duplicate: Analyze a specimen from the beginning of the run towards the end of the day's run. Result must agree within ± 0.2 of the previous value.

Note: The analyzer is programmed to begin a 3 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 3 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 2 hours the analyzer will shut down automatically.

(6) Prepare samples for analysis:

Controls: Thaw aliquots and vortex briefly. Using the autodilutor, prepare hemolysates of each control. Wipe the probe tip after drawing up sample and again after dispensing into a labeled vial. Place the sample vials in the rack.

Preparing patient specimens:

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 (less than 1.0 mL whole blood in tube). Using the autodilutor, prepare hemolysates. 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 150 uL.

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

(7) Place racks in ascending order into the sample loader. 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, then analyze samples at 1.6 minute intervals. The analyzer aspirates 4 µL of whole blood. The whole blood sample is automatically diluted approximately 1:200 and introduced into the assay line. When the assay begins, the sample rack is transferred and continuous sampling starts and continues until an empty rack on the loader is detected.

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

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 3 minutes. Once it has started, always allow the WASH sequence to go to completion! If washing is insufficient, column lifespan will be reduced and the result for the next sample could be affected.

(8) If there is no further input from the operation panel while the analyzer is in STAND-BY mode, after 2 hours (Off Time setting), the analyzer shuts itself off automatically.

9. REPORTABLE RANGE OF RESULTS

REPORTABLE RANGE: 3.1 – 19.0 %
Report results falling outside this range as <3.0 or >19.0 %.

10. QUALITY CONTROL (QC) PROCEDURES

Two levels of glycated hemoglobin control (Normal and Elevated) are analyzed in duplicate (or more) with each batch. Controls must also be analyzed at the start and end of every day, after calibration, maintenance, and as needed for troubleshooting.

Controls are prepared from whole blood drawn from a normal (Normal) and a diabetic (Elevated) individual (See paragraph below for preparation instructions). Stable indefinitely stored at –70^{o}C. Controls should be diluted with Hemolysis & Wash Solution to obtain a Total Area on the chromatogram in the range of 500-4000. If the value of one or more control specimens is out of the acceptable range, recalibrate the system and reanalyze 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^{o}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^{o}C freezer.

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

AMR
Analytical measurement range (AMR) procedure or Linearity Check is performed every 6 months. Results must be analyzed in duplicate and be within 15% of the assigned values. Document on linearity check sheet in G8 Documentation binder.

New Lot Verification
If a new lot number of **buffer** is installed, analyze 5 duplicate samples along with the controls at the beginning of the run. Recalibration is not required. Results should agree within 0.2%. Record these results on the Rgt/Column Change Sheet in the G8 Documentation binder.

If a new lot number of **column** is installed, analyze three whole blood specimens to prime the column. Calibrate, then analyze 5 duplicate samples along with the

controls at the beginning of the run. Results should agree within 0.2%. Record these results on the Rgt/Column Change Sheet found in the G8 Documentation binder

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 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, analyzing 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. Documentation is in the G8 Documentation binder .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.**

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.

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. Consult with lead tech and store samples appropriately until resolution of issue.

12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS

Total Area
Dilution studies demonstrate that the assay is linear from a Total Area of 500 to 4000.

Optimum Total Area is 700 to 3000. Do not report an A1C result if the total area is less than 500 or greater than 4000.

For areas greater than 4000, a dilution can be made using a smaller sample size or more diluent to bring the area within acceptable range, and reanalyzed. For areas less than 500, a dilution can be made using a larger sample size or concentration by centrifugation and making a 'normal' dilution, and reanalyzed.

Abnormal Red Cell Survival
The life span of red blood cells is shortened in patients with hemolytic anemias, and the actual life span depends upon the severity of the anemia. As a consequence, specimens from such patients may exhibit decreased glycohemoglobin levels compared to patients with normal 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.

Hemoglobinopathies

- HbA1c results cannot be reported in patients with heterozygous Hemoglobinopathies in which the non-A hemoglobin elutes **before** the A0 peak. In such situations, the results may be falsely increased or decreased depending upon the retention time of the abnormal hemoglobin. If the hemoglobin variant elutes independently of the SA1c peak, but before the A0 peak, it will cause a false decrease in the SA1c result. This may occur with HbE.
- Peak areas of hemoglobin's eluting **after** the A0 are not included in the calculations of Total Area on the G8 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.
- Glycemic monitoring for any patients displaying any homozygous hemoglobin (other than HbAA) such as HbSS, HbCC or the double heterozygous SC, cannot be performed using SA1c because there is no hemoglobin A present. Alternative testing is mandatory for these types of patients.

Hemoglobin adduct interferences
Various substances other than glucose can form adducts with hemoglobin, thereby altering its charge characteristics. Falsely elevated results can occur if these adducts co-elute with the stable HbA1c. Studies on the Tosoh Automated Glycohemoglobin Analyzer HLC-723G8 in the Variant Analysis

Mode indicate that the carbamylated hemoglobin (as seen in uremia) and acetaldehyde hemoglobin (as seen with alcohol consumption) derivatives elute in the labile fraction. No interference in stable HbA1c results from these derivatives has been observed up to concentrations of 0.25 g/L of carbamylated hemoglobin and 0.25 g/L of acetylated hemoglobin

Interferences:
Variant hemoglobin interference studies were also conducted on diabetic and nondiabetic samples spiked with a high human specimen for HbF, and commercially available control material for HbAE, HbAD, HbAS, and HbAC. **HbAE** is not distinguished from the other peaks, and if present will interfere with sA1c measurement.
No interference was observed for **HbF** up to a concentration of **10 %**.
No interference was observed for **HbAD, HbAS(**approximate retention time 1.2) **and HbAC** (approximate retention time 1.3) up to a concentration of **45%** since these variants elute after the A0 peak.

Interference studies were conducted with diabetic and non-diabetic samples. They were spiked with increasing amounts of glucose, bilirubin, triglycerides, sodium cyanate, acetylsalicylic acid, acetaldehyde and EDTA. Interference was determined as a variance greater than the (assigned value x 1.00 +/- 5%) Labile A1c, as indicated by glucose concentrations up to 1000 mg/dL does not interfere with the assay.

Icterus, as indicated by free and conjugated bilirubin concentrations up to 20 mg/dL does not interfere with the assay.

Lipemia, as indicated by triglyceride concentrations up to 1000 mg/dL do not interfere with the assay.

Concentrations of up to 25 mg/dL sodium cyanate (carbamylated Hb) do not interfere with the assay.

Concentrations of up to 25 mg/dL acetaldehyde (alcohol) do not interfere with the assay.

Concentrations of up to 50 mg/dL of acetylsalicylic acid (aspirin) which forms acetylated Hb, 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 6.5%. 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; PROTOCOCOL FOR REPORTING CRITICAL CALLS (IF APPLICABLE)

NHANES SA1c% results are entered unto 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

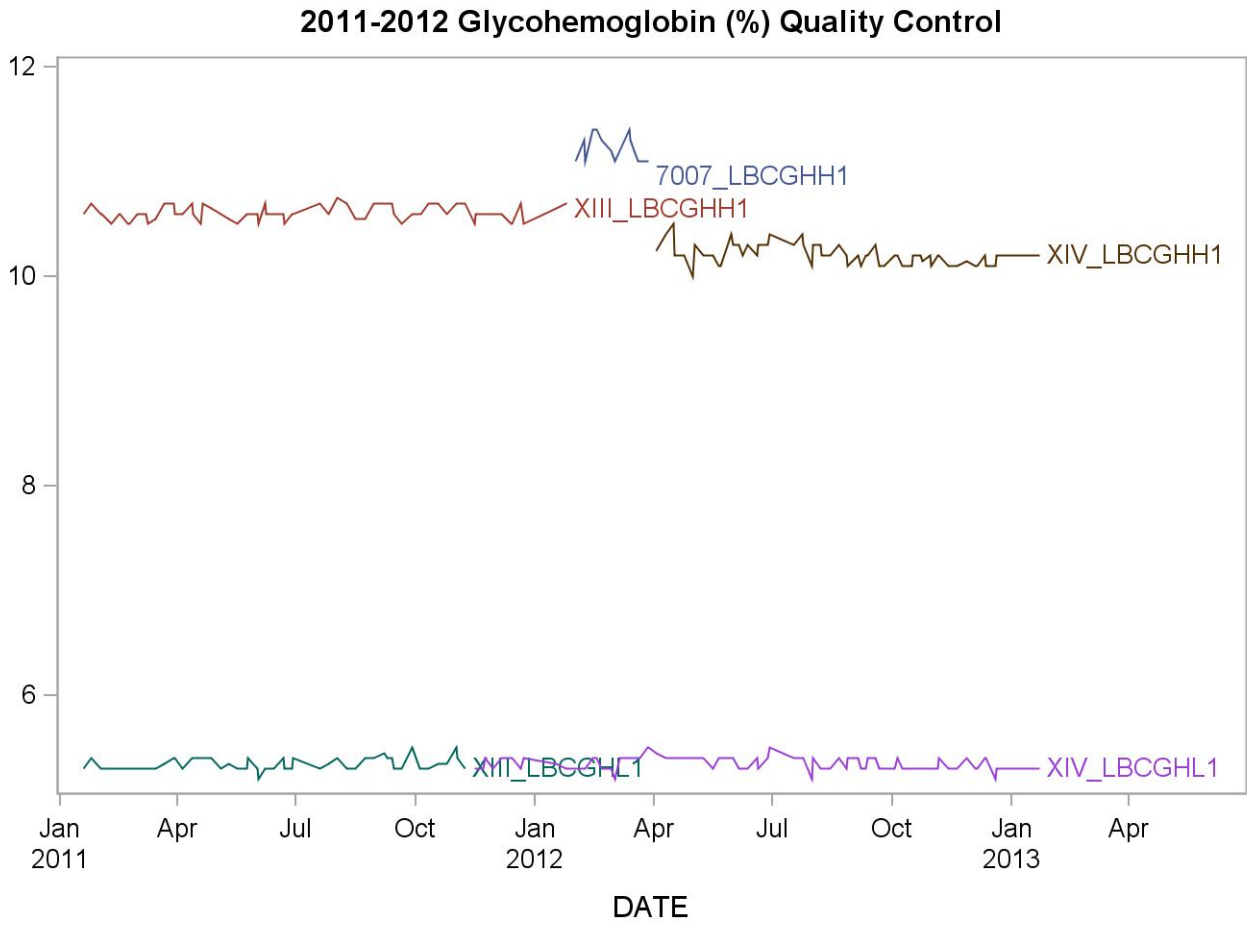
All shipments are recorded on the NHANES Shipping Log upon receipt. Actions taken during the course of analysis, result reporting, and specimen retention are also recorded on the log.

19. SUMMARY STATISTICS AND QC GRAPHS

See following pages.

Summary Statistics for Glycohemoglobin (%)

LBCPNME	Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
LBCGHH1	XIII_LBCGHH1	84	19JAN11	25JAN12	10.6131	0.0773	0.7
LBCGHL1	XIII_LBCGHL1	69	19JAN11	08NOV11	5.3449	0.0607	1.1
LBCGHL1	XIV_LBCGHL1	95	15NOV11	22JAN13	5.3516	0.0650	1.2
LBCGHH1	7007_LBCGHH1	15	01FEB12	28MAR12	11.2133	0.1246	1.1
LBCGHH1	XIV_LBCGHH1	65	03APR12	22JAN13	10.2046	0.1022	1.0



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