

Laboratory Procedure Manual

Analyte: **Glycohemoglobin**

Matrix: **Whole Blood**

Method **Tosoh G8 Glycohemoglobin Analyzer**

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

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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_H	LBXGH	Glycohemoglobin (%)

1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

The Tosoh Automated Glycohemoglobin Analyzer HLC-723G8 is intended for *in vitro* diagnostic use for the quantitative measurement of % hemoglobin A1c (HbA1c) in whole blood specimens. HbA1c measurements are used in the clinical management of diabetes to assess glycemic control.¹ This test is also used as an aid in the diagnosis of diabetes identifying patients who may be at risk for developing diabetes.²

The procedure is specifically designed for the Tosoh Automated Glycohemoglobin Analyzer HLC-723G8 equipped with appropriate software, TSKgel G8 HSi Column, Elution Buffers, and Hemolysis & Wash Solution.

The analyzer uses non-porous ion exchange, high performance liquid chromatography (HPLC) and microcomputer technology to quickly and accurately measure the HbA1c as a percentage of the total amount of hemoglobin present in the sample.

Summary and Explanation of the Test

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

Although a fasting blood glucose measurement gives the clinician information about the patient's status over the last twelve hours, the stable HbA1c offers a more accurate indication of the patient's long-term diabetic control over the last two to three months.

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

HbA1c is nonenzymatically synthesized in two steps:

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).

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

In the past, accurate measurement of SA1c was possible only after removing LA1c by pretreatment. The Tosoh Automated Glycohemoglobin Analyzer HLC-723G8 can individually resolve SA1c and LA1c on the chromatogram without manual pretreatment, allowing accurate measurement of SA1c directly.

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 with Hemolysis & Wash Solution, and then injects a small volume of this specimen onto the TSKgel G8 Variant HSi Column. Specimens may also be diluted offline using the dilution procedure below. Separation is achieved by utilizing differences in ionic interactions between the cation exchange group on the column resin surface and the hemoglobin components. The hemoglobin fractions (designated as A1a, A1b, F, LA1c+, SA1c, A0, and H-V0, H-V1, H-V2) are subsequently removed from the column by performing a step-wise elution using the varied salt concentrations in the 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. Specimens that show variant peaks are subsequently assayed by a boronate affinity HPLC method (refer to separate *ultra*² SOP).³

2. SAFETY PRECAUTIONS

Follow all procedures and policies in the University of Missouri’s 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.

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

(1) Patient Preparation:

No special conditions such as fasting or special diets are required.

(2) Type:

a. Collect whole blood specimens in vacuum collection tubes containing EDTA and mix thoroughly.

b. As per manufacture instructions; specimens may be stored up to fourteen days at 2-8°C before analysis. Specimens may be stored

up to twenty four hours at room temperature (10-25°C) before analysis.² However, a stability performed in-house and published through Diabetes Technology & Therapeutics demonstrated the below WB sample stability;^{5,6}

Room Temperature: 7 days **4°C: 21 days**⁵

Frozen (- 20°C);(-70°C): 7 days ⁶; 1 year ⁷

The minimum volume required for analysis directly from collection tubes is 1 mL of whole blood. Whole blood samples as small as 50 µL may be used when appropriate sample cup and software options are selected.²

- c. Specimens collected using the Bio Rad HbA1c Capillary Collection System(P/N 270-2167, (Bio Rad Laboratories, Hercules, CA) are also acceptable.³ These contain an additional stabilizer, and as such are stable for up to six months at 2-8°C and 2 weeks at room temperature.
 - d. Specimens are delivered to the Diabetes Diagnostic Laboratory, Room M764 by Pathology Immediate Response (IR) Processing. Each specimen must arrive in the laboratory labeled with a unique accession number generated by the Cerner Pathnet computer system, unless downtime procedures are in effect.
- (3) Unacceptable specimen criteria:

- 1. Clotted samples.
- 2. Unlabeled samples (specimens are to be labeled with patient name, rec. number, accession, test order, and transferring lab name).
- 3. Specimens not collected in EDTA or Bio Rad HbA1c Capillary Collection tubes.

The below must be followed for unaccepted specimen collections:

- 1. Record all unacceptable samples in Unacceptable Pathology Samples Received Log.
- 2. Attempt to find an acceptable sample to backorder a HbA1c from.
- 3. If one is found, backorder the HbA1c using the ordering information from the problem sample.
- 4. If a sample is considered unacceptable and a suitable replacement is not available, notify the clinic or unit clerk by phone and email the requesting physician.
- 5. Return rejected specimens by calling IR processing (882-1242).

(4) Handling Conditions:

- a. Samples are to be kept refrigerated at 4°C immediately after collection.
- b. Transport under refrigerated conditions.
- c. Once received and prepared for analysis, specimens are to be immediately returned to 4°C storage where they are to

be kept for one week before being discarded. If longer term storage is necessary, specimens may be frozen and stored at -70°C (DO NOT FREEZE SAMPLES AT -20°C).⁵

5. PROCEDURES FOR MICROSCOPIC EXAMINATION

Not applicable for this procedure.

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

(1) Equipment:

- a. Tosoh G8 Glycohemoglobin Analyzer (Tosoh Bioscience, Inc., South San Francisco, CA.)

Parameter	Setting
Wavelength	Sample: 415nm Reference: 500nm
Column Temperature	25 ° C
Injection Interval	1.6 min
Calibration	Two-point
Working Temperature	15°C ~ 30°C

- b. Hamilton Autodilutor Model Microlab 500 or 600 with 2.5 mL and 25 µL syringes (Hamilton, Reno, NV)
- c. Microsoft Windows Compatible Computer capable of running Microsoft Internet Explorer, Cerner Pathnet software, and G8 Data Management Software.
- d. G8 Data Management Software (Tosoh Bioscience, Inc., South San Francisco, CA)
- e. Rainin Variable Volume Pipettes (Mettler Toledo Oakland, CA) in 0.5-10, 2-20, 20-200, and 100-1000 µL volumes.

(2) Equipment Maintenance

- a. Tosoh Analyzer System—Routine maintenance
 1. Column pre-filters – Replace the filter element if the pressure is greater than the pressure level that is indicated on the column inspection report +4 MPa or after 400 injections.¹ At least 5 prefilters should be on hand at all times. Analytical column—Change after 2500 injections. ¹ At least two spare columns are to be kept available at all times.
 2. Record all routine maintenance in the Tosoh G8 Diary.

- b. Tosoh Analyzer System—Periodic/Preventative Maintenance
 - i. Replacing Buffers: Solution of Hemolysis Wash
 - 1. Confirm that the lot #'s match the corresponding buffers. If the analyzer is not in STAND-BY mode, press the STOP key and wait until 'STAND-BY' appears on the Status screen.
 - 2. Remove old buffer bag from analyzer and replace with a new bag. Be sure that the color of the connection tubing matches the color of the buffer bag label.
 - 3. Write the date opened and an expiration date three months into the future on each bag or bottle as it is opened.
 - 4. From the MAIN screen, select **MAINTE**, then **REAGENT CHANGE**. Once the buffers have been correctly installed, select the appropriate buffer, and then press **CHANGE**.
 - 5. Record new lot information in G8 Data Management software, daily diary sheet, and buffer record in G8 binder.
 - ii. Removing air from the buffer lines
 - 1. Air can enter the fluid lines if a buffer bag runs dry or after long-term shutdown. The following procedure removes air from the lines:
 - 2. From the MAINTE screen, press **REAGENT CHANGE** key.
 - 3. Highlight the key(s) for the reagent(s) to be primed.
 - 4. Press **PRIME** key. The confirmation message will be displayed. If everything is ready press the **OK** key. The reagent(s) in the analyzer fluidics will automatically be replaced with fresh reagent. The operation is complete when the "**PRIMING...**" display disappears. Approximately 5 mL of each reagent will be consumed when **PRIME** is executed.
 - 5. Pump buffers and verify pressure. Repeat if necessary.
 - iii. Removing air from the pump
 - 1. During pumping, if the pressure does not rise, air may be present on the outlet side of the pump.
 - 2. Use the following procedure to remove the air.
 - 3. Verify analyzer is in STAND-BY mode.

4. If the analyzer is not in STAND-BY mode, press the **STOP** key and wait until 'STAND-BY' appears on the Status screen.
 5. Press the **REAGENT CHANGE** key on the MAINT screen.
 6. Press the **DRAIN FLUSH** key.
 7. The following message will be displayed requesting that the drain valve be opened: "Open the door on the left side of the analyzer and turn the drain valve 90 degrees in the counterclockwise direction to open the valve."
 8. Turn the valve ONLY 90 degrees counterclockwise.
 9. Press the **OK** key.
 10. A confirmation message will appear. Ensure the drain valve is open. Press the **OK** key again.
 11. Air stuck in the pump will automatically be removed. This procedure takes approximately 7 minutes to complete and is finished when the "**FLUSHING...**" message disappears.
 12. A message will be displayed requesting that the drain valve be closed. Turn the valve back 90 degrees in the clockwise direction to securely close it.
 13. Press the **OK** key.
 14. Press the **EXIT** key to return to the main screen second page. Press the **PUMP** key.
 15. If a pressure of within the acceptable range for the filter is displayed in the HbA1c mode with no pressure fluctuation, air removal is complete. Press the **PUMP** key again to stop the pump motor. If the pressure does not rise 5Mpa or is unstable, stop the pump and repeat the air removal procedure again.
- iv. Replacing the Filter Element
1. Replace the filter element after 400 injections, when the pressure exceeds limits established upon installation of the column, or it is reasonable that the injection limit will surpass 400 during an analytical batch.
 2. Verify analyzer is in STAND-BY mode.
 3. If the analyzer is not in STAND-BY, press the **STOP** key and wait until 'STAND-BY' appears on the Status screen.
 4. Open the door below the display.
 5. Confirm that the SV1 key is open (O) on the second page of the main screen.
 6. Remove the filter outlet (peek) tubing from the top of the filter assembly.

7. Loosen the top of the filter holder assembly by turning it counterclockwise. Remove the filter holder by pulling it straight out.
 8. 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 gray colored surface is the outlet (up) side.
 9. Firmly tighten the top of the filter holder assembly by hand until no further tightening is possible.
 10. Slide outlet tubing unit it extends $\frac{1}{4}$ inch past the end of the tubing. Connect the outlet side tubing.
 11. Press the **PUMP** key again 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.
 12. Press the **PUMP** key to stop the pump.
 13. Reset filter counter to 0 on the REAGENT CHANGE screen.
 14. Record change on daily diary sheet.
- v. Column Replacement
- Replace the column in the following situations.
1. Replace column after 2500 injections.
 2. When the pressure is more than what is indicated on the column inspection report + 4 MPa and is not reduced by filter replacement.
 3. When peaks on the chromatogram (particular the shaded SA1c peak) have become broad or broken in two fractions.¹
 4. When assay results for quality control samples are consistently out of assigned ranges even after re-calibration.
 5. When the CALIB ERROR persistently occurs. Please contact Technical Support if the above issues are not resolved after column replacement.
- vi. Replacing the Column
- Replace the column if column maintenance (see above) does not solve the problem and if the column exceeds 2500 injections, according to the following procedure. Verify analyzer is in STAND-BY mode.

- a. If the analyzer is not in STAND-BY mode, press the **STOP** key and wait until 'STAND-BY' appears on the Status screen.
- b. Remove old column.
- c. Open the front doors of the analyzer. Release latch and open the column oven. Unscrew column connections and remove used column.
- d. Confirm that the SV-1 key is open (O) on the MAIN screen, (second page).
- e. Slide the inlet tubing unit it extends $\frac{1}{4}$ inch past the end of the fitting. Connect the new column to the pump (right) side only. Take care that the flow arrow on the column indicates flow right to left. Press the **PUMP** key allowing buffer flow into the column. When the buffer begins to flow from the open end of the column, press the **PUMP** key stop the flow.
- f. Connect detector tubing to outlet (left) side of column. Slide the outlet tubing until it protrudes $\frac{1}{4}$ inch past the end of the fitting. Insert the outlet tubing into the left side of the column. Screw the fitting finger tight.
- g. Check for leaks. Press the **PUMP** key to start the pump and confirm there is no fluid leakage.
- h. Check for fluid leaks at the connections. If leaks occur, tighten fittings.
- i. Verify that pressure stabilizes. The pressure should rise to the pressure level that is indicated on the column inspection report + 4 MPa. If leaks occur, tighten fittings.
- j. Reference pressure and limits should be recorded onto a label attached to the instrument. This label should include the data, reference MPa, low and high MPa limits, and column serial number.
- k. After verifying connections are secure, stop the pump by pressing the **PUMP** key.
- l. Close column oven.
- m. Close front doors of the analyzer.
- n. After connecting a new column, reset (zero) to the column counter in the REAGENT CHANGE screen.
- o. Record change on daily diary sheet.
- p. Run at least three whole blood samples to prime the new column. Verify that the retention time for the SA1c peak is between 0.57 – 0.61 minutes. The ideal the retention time for SA1c is 0.59 minutes.⁵
- q. If necessary, adjust the flow rate to match the retention time for the SA1c peak on the reference chromatogram included with the column.

- r. Once the retention time matches within +/- 0.2 min, print off the chromatogram and submit it along with the included chromatogram from the manufacturer to the supervisor for approval of the use of the column.
 - s. In the event of column lot change, all reagents need to be replaced to lots corresponding to the new column lot. A comparison needs to be done between the old and new lots of columns/reagents (n=40). Comparison must meet these criteria:
 - i. XY plot (current lot on x-axis) with linear regression performed.
 - ii. Slope = 1.0 +/- 0.1
 - iii. Intercept = 0.0 +/- 0.1
 - iv. $R^2 > 0.98$
 - v. Prepare Bland/Altman plot. 95% CI of the differences between x and y within 0 +/- 0.5% HbA1c. Overall mean bias within +/- 0.2% HbA1c. If any outliers (>1% HbA1c difference between X-Y) occur, investigate further.
- vii. Replacing printer paper.
1. Lift the printer cover (upper lid) to the back to open.
 2. Push the paper holding lever down to the very front and wrap the remaining paper onto the roll.
 3. Lift the roll up and remove the mandrel.
 4. Insert the mandrel into the new roll with attention to the direction.
 5. Return the paper holding lever to the very back and insert the paper into the printer. Press the feed switch to feed the paper.
 6. Check for twisted paper. If the paper is twisted, push the paper holder lever to the front, adjust the paper, and return the lever to the back.
- viii. Replacing the sampling needle.
1. Replace the needle if it is bent or broken. Although needle replacement is normally done by field service personnel, the procedure below may be performed by the operator.
 2. Put on protective clothing (goggles, gloves, etc.) and take care not to touch the end of the sampling needle during handling.
 3. Press the POWER key to switch off the analyzer.
 4. Use a screwdriver to remove the sampling cover screws.
 5. Remove the sampling needle cover.

6. The sampling needle unit is located behind this cover. Grasp the upper part of the sampling needle unit by hand and slowly pull the unit forward as far as possible.
 7. A small volume of reagent may leak during needle replacement. Place a tissue or plastic pad under the sampling needle tip to absorb any leakage.
 8. By hand, loosen and remove the tubing connected to the 3-way block.
 9. Remove the screws on the upper section of the sampling needle. Be careful not to drop the screws or the holding plate inside the machine during this operation.
 10. Remove the screws that hold the guide through which the tubing passes.
 11. Slowly lift up the sampling needle to remove it. Place immediately into a sharps container.
 12. Insert the new sampling needle with the bevel facing forward. The sampling needle must be positioned with the bevel facing forward or the needle will not correctly dilute the sample.
 13. Secure the holding plate with the screws.
 14. Pass the tubing through the guide, secure with the screw, and securely connect the tubing to the 3-way block.
 15. Move the sampling unit back and forth and confirm that the tubing does not catch. If necessary, loosen the screws and change the guide direction to prevent the tubing from being obstructed. Push the sampling unit back; close the blue cover by following the above procedure in reverse. Secure the screws.
 16. Turn on the Main Power Switch. Press the **POWER** key on the control panel and allow the analyzer to complete the WARMUP process then to the STAND-BY state.
 17. Assay 3 whole blood samples to confirm the sample is aspirated correctly. The Total Area for these samples should be approximately the same as it was before the sampling needle replacement.
- ix. Adjusting the Flow Rate.
The flow factor is generally 1.00 mL/min, but can be 1.03 mL/min dependent on instrument factory setting. ⁶ The flow factor should only be adjusted +/- 0.05 of the default factory setting. ¹
- x. Instrument Preventative Maintenance (PM)

1. A monthly PM is performed by a trained member of our DDL staff using the monthly PM checklist. The completed checklist is reviewed by the lab supervisor for any problems.
2. For every 20K injections, a PM is performed by a Tosoh Service representative. ⁶

xi. Pipette Preventative Maintenance

1. Hamilton Autodilutor 500/600
 - a. Annually, complete the Carryover procedure according to Autodilutor Carryover SOP.
 - b. Annually, verify calibration of the device according to Autodilutor SOP.
 - c. Instrument should be cleaned with disinfectant daily and periodically the internal mechanisms should be cleaned and oiled.
2. RAININ Pipettes
 - a. After each use, the pipette should be wiped with disinfect with soaked gauze.
 - b. Annually disassemble, clean, and reassemble pipettes.
 - c. Annually verify calibration using the procedure found in the Clinical SOPs binder.

(3) Materials:

- a. Reagents—All reagents are supplied by Tosoh Bioscience (South San Francisco, CA)

The following components are available for the G8 Analyzer Variant Analysis Mode. Part #	Description	Packaging
021560	Tosoh Automated Glycohemoglobin Analyzer HLC-723G8	1 each
021561	90 Sample Loader	1 each
021562	290 Sample Loader	1 each
021955	TSKgel G8 Variant HSi	1 each
021956	G8 Variant Elution Buffer HSi No.1 (S)	1 × 800 mL
021957	G8 Variant Elution Buffer HSi No.2 (S)	1 × 800 mL
021958	G8 Variant Elution Buffer HSi No.3 (S)	1 × 800 mL
018431US	HSi Hemolysis & Wash Solution (L)	1 × 2000 mL
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
020101	Cup adapter for Tosoh sample rack	10each

b. Other Materials

- i. Powder free nitrile exam gloves. (Fisher Scientific, Waltham, MA)
- ii. Oxyvir disinfectant. (AHP Technology, Sturtevant, WI))
- iii. Gauze Sponges 4x4 not sterilized (Fisher Scientific, Waltham, MA)
- iv. Kim Wipe lintless tissues. (Fisher Scientific, Waltham, MA).

(4) Storage Requirements:

- a. Unopened Elution Buffer 1, 2, and 3 are stable at room temperature until the expiration date printed on the label. After opening, Elution Buffers packaging are stable for three months. Store at 4-30 °C.

- b. 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.
- c. 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. Replace column after 2500 injections.
- d. Reagents must be brought to room temperature prior to use.

(5) Reagent labeling

Reagents, calibrators, controls, and solutions should be traceably identified to indicate the following:

- a. Content and quantity, concentration or titer
- b. Storage requirements.

The below should be followed for working reagents;

- a. Preparation date or opened date and the identity of the preparer.
- b. Tech's initials.

7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

(1) Calibration Preparation:

- a. Pooled Low Calibrator #5 (PLC5)
 - i. Single level calibrator prepared from K₂EDTA whole blood drawn by venipuncture from four non-diabetic individuals.
 - ii. The blood specimens were pooled, dispensed in 30uL aliquots into 400uL microtubes at 2-8°C and frozen the same day. Part of the PLC5 aliquots were frozen at -70°C, the remaining aliquots were placed in a cryogenic (liquid nitrogen) tank at -196°C.³
 - iii. %HbA1c value was assigned by twenty interassay determinations along with the previous lot of calibrator. The assigned value of PLC5 is 5.27% HbA1c.
- b. Pooled High Calibrator #5 (PHC5)
 - i. PHC5 was prepared from pooled EDTA whole blood by Aalto Scientific.
 - ii. PHC5 is aliquoted in 250 uL aliquots. Part of the PHC5 aliquots were frozen at -70°C, the remaining aliquots were placed in a cryogenic (liquid nitrogen) tank at -196°C.
 - iii. %HbA1c value was assigned by twenty interassay determinations along with the previous lot of calibrator. The assigned value of PHC5 is 11.88% HbA1c.
- c. Preparation and Stability
 - i. One 30uL of PLC5 is thawed from -70°C each day.

- ii. One 250ul aliquot of PHC5 is thawed at the beginning of each week, and the date that it was thawed is written on the vial. PHC5 is good for five days after thawing when stored at 4 °C, but acceptability is based solely on the quality of chromatography.
- iii. Three 1.5ml sample vials of calibrator are prepared in the same manner as an unknown specimen as described in PROCEDURE-STEPWISE section 4.
- iv. Calibrators are stable for up to two years at -70°C, and up to five years or longer in liquid nitrogen. When controls and calibrators are transferred from liquid nitrogen to -70°C, an expiration date is written inside the lid of the box transferred that is two years from the transfer date.
- v. Refer to specimen section for stability requirements.

(2) Frequency:

Calibration is to be performed:

- a. Daily prior to the first analytical batch of the day on that instrument.
- b. If drift in QC is observed.
- c. When controls values are out of range.
- d. After a column replacement.
- e. After analyzer maintenance.

Refer to Operator's Manual for additional troubleshooting advice.¹

(3) Calibration Procedure:

- a. Verify that there is sufficient volume of Elution Buffers, Hemolysis & Wash Solution and at least 400 µL of each calibrator in the sample cup.
- b. Check analyzer status.
- c. If analyzer is in Standby mode, proceed.
- d. On Main screen make sure CALIB is reversed highlighted.
- e. Place the sample vials in the rack and tubes with PLC5 in position 1 (on the left) and PHC5 next to it in position 2.
 1. Press the START key to begin the calibration.
 2. The analyzer measures Calibrator 1 three times and Calibrator 2 two times for a total of 5 times. The analyzer discards the first measurement, and uses the remaining four measurements to calculate the slope and the intercept. Patient sample results following calibration will be calculated using the new factors.

*Current Calibrator value assignments can be found in the Quality Control Binder located in room M771.

(4) Calibration Acceptability Criteria:

The analyzer has a two-point automatic calibration function for stable HbA1c (SA1c). When the analyzer processes calibrators, it calculates the slope and the intercept from a linear regression equation to determine quantitative results from patient samples and controls. When the calibration procedure is completed, the analyzer automatically accepts or rejects the calibration results. If the calibration is unsuccessful, recalibration is required.

A Calibration Error message appears and the run aborts if;

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

**8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS;
INTERPRETATION OF RESULTS**

(1) Special Safety Precautions:

- a. Gloves, lab coat, and safety goggles are required for handling all human blood specimens. When working in the biological safety cabinets, appropriate PPE shall be donned.
- b. Vials containing human blood are only to be opened in a biological safety cabinet with the sash in the correct position.
- c. All plastic tips, sample cups, gloves, etc. that contact blood are considered contaminated and are to be placed in a biohazard waste container.
- d. All hoods, telephones, doorknobs and work surfaces are wiped down with Oxyvir disinfectant or 10% bleach at least one time during each work shift. Any area in which blood is spilled is also to be cleaned and disinfected immediately with Oxyvir disinfectant or 10% bleach following the SOP for biohazardous spills.

All healthcare personnel shall routinely use appropriate barrier precautions to prevent skin and mucous membrane exposure when contact with blood or other body fluids of any patient is anticipated. All products or objects that come in contact with human or animal body fluids should be handled, before and after cleaning, as if capable of transmitting infectious diseases. Wear appropriate Personal Protective Equipment (PPE), including facial protection, gloves, and protective clothing.

Dispose of all biological samples and diluted specimens in a biohazard waste container at the end of analysis.
Dispose of all liquid hazardous waste in properly labeled hazardous waste container

(2) Initial processing of Pathology specimens

- a. All specimens must be verified as received by the laboratory on the Cerner Pathnet computer system prior to be analyzed. Use the following procedure.
 1. Login to Pathnet.
 2. Open Specimen Log-In
 3. Select the Accession Radio Button
 4. Click on the Retrieve button
 5. Select UH Diagnostic Diabetes as the Location (after 1st use, this will default to this location upon subsequent log-ins)
 6. Scan the barcode on each tube to be logged in
 7. After scanning all tubes to be logged in, verify only tubes with HbA1c as orders are selected, and click the log-in button.
 8. After specimens are finished logged, close the window.
 9. Place specimens in the rack designated for samples to be analyzed in the refrigerator immediately after completing initial processing in numerical order.

(3) Instrument setup for the Tosoh Analyzer System.

- a. Check levels of buffers 1, 2, and 3 and wash solution, making sure there is sufficient volume to complete the assay. Add more buffer as necessary. All changes in lot numbers of buffers are to be recorded on the Tosoh Diary worksheet, and in the Maintenance section of the Tosoh Diaries Binder.
- b. Analyzer should be in STANDBY mode prior to beginning analysis.
- c. Record # of injections on column. (Listed on MAIN screen)
- d. Record # of injections on filter. (Listed on MAIN screen.
- e. Check pressure listed on the MAIN screen and record.
- f. If pressure is greater than +4 MPa over the reference value listed on the inspection report, replace the filter.

- g. If pressure is less than the reference values, check for leaks and secure leaking fittings near the filter assembly and column.
- h. Check temperatures, including the minimum and maximum, for the room (M764), the refrigerator in M764, and the ultralow freezer in M764 and record the values on the weekly environmental conditions checklist. If values are found to be outside of limits, the supervisor is to be notified and the course of action documented.
- i. Check for air bubbles & leaks:
During warmup, check the tubing connections for leaks, particularly the filter and column inlet and outlet sections. Tighten connections if a leak is found. Record check in the Tosoh Diary.
- j. Check calibration status.
Verify that the MAIN screen has the CALIB button reverse highlighted.
- k. Check printer paper.
Open the printer lid and check the remaining paper. Be sure that there is sufficient paper to complete the assay. Replace with a new roll of paper if needed.
- l. Check Flow rate:
From the MAIN screen, press the **MENU** key, then **PARAMETER**, and **▼**. Record the flow factor in the Tosoh Diary.
- m. Controls at end of run:
Be sure that there are low and high level controls at the end of the assay.
- n. Verify the Tosoh G8 Reporting Software program is open.

(4) Sample preparation – For Controls, Calibrators, and specimens requiring pre-dilution

- a. Using a Hamilton Autodilutor, prepare control and calibrator hemolysates by diluting 10ul of well mixed whole blood with 0.990 mL hemolysis reagent in the sample vial. Use the following procedure:
 - 1. Wipe outside of tip with gauze wetted with distilled water.
 - 2. Insert tip of autodilutor into blood specimen and press the button on top of the handle to draw 10ul of specimen into the tip.

3. Insert tip into corresponding 1.5mL sample cup and press button again to dispense sample and reagent into sample cup.
4. Wipe outside of tip again with gauze wetted with distilled water.
5. Repeat procedure for all QCs and specimens not suitable for direct sampling on the instrument from the primary tube.

b. Load calibrators.

Place the sample vials in the rack with PLC5 in position 1 (on the left) and PHC5 in position 2.

c. Load controls.

Controls are performed at the beginning and at the end of a run and every 19 samples.

1. Place controls in the rack with WB25 (low) in position 3 and WB 28 (high) in position 4.
2. Next, load low controls every 19 samples, WB25 followed by WB28.
3. Place WB25 and WB 28 in the end positions.

d. Load samples.

1. Mix each sample by gently inverting each capped sample tube.
2. Place capped sample tubes in the rack in order from left to right. If you are using barcoded tubes, verify that the labels face the analyzer.
3. Position each rack in the rack guide, starting with the right side.
4. Place a blank rack after the last rack of samples. The blank rack serves as an end marker.

(5) Operation of the Tosoh Analyzer System.

- a. Press the **POWER** button. (Already in standby when performing instrument setup.)
- b. Follow the calibration procedure in the CALIBRATION section of this manual.
- c. Press the **START** button.
- d. Check chromatograms from normal and abnormal QC specimens in positions 1, 2, 3 and 4.
- e. When measurement ends, the analyzer washes the column by pumping buffer for 15 minutes, and then enters STAND-BY mode.
- f. After run is completed, verify and input, when necessary, accession numbers for each individual specimen in the G8 Reporting Software under the heading "barcode" using the accession number generated by the Cerner Pathnet computer system.

- g. Examine individual chromatograms.
- i. Each chromatogram should include six peaks identified as A1A, A1B, F, LA1C+, SA1C, and A0.
 - ii. All peaks should be clearly resolved.
 - iii. The acceptable retention time for SA1c is 0.57 – 0.61.¹
 - iv. The acceptable retention time for A0 is 0.87 – 0.91.¹
 - v. The acceptable range of TOTAL AREA is from 500 to 4000. However, optimal results are obtained in the TOTAL AREA (TA) range from 700 to 3000. Do not report results with a TA < 500 and > 4000. Low or high hematocrit samples may display Total Area above or below the Total Area linear reportable range;
 - Samples that exhibit TA < 500; reassay the specimen using less Hemolysis & Wash Solution and centrifuge lightly to yield a TA between 500 – 4000.¹
 - Samples with TA > 4000; reassay the specimen using more Hemolysis & Wash Solution.¹

There is not absolute guideline for correlating a hematocrit with the dilution needed. Matching the color of the diluted blood with the color of the diluted calibrator will ensure a good TA.¹
 - vi. Repeat any specimens with %HbA1c values less than 4.0% or greater than 14.0% for verification.³
 - vii. Any samples which show a deterioration peak (extra peak between the SA1C and A0 peaks usually designated as P00 or P01) are to be marked “X-PK”. Rerun by Trinity *ultra*² affinity method and report the *ultra*² HbA1c result. Refer to *ultra*² HPLC SOP.
 - viii. Samples with heterozygous HbD will exhibit an additional peak or peaks after the A0 peak, and the instrument will designate the main peak HV-0, and minor peaks Pxx. The chromatogram is labeled with “HbAD”, and the %HbA1c cannot be reported for these specimens. Rerun by Trinity *ultra*² affinity method and report the *ultra*² HbA1c result. Refer to *ultra*² HPLC SOP.
 - ix. Samples with heterozygous HbS will exhibit an additional peak or peaks after the A0 peak, and the instrument will designate the main peak HV-1, and minor peaks Pxx. The chromatogram is labeled with “HbAS”, and the %HbA1c cannot be reported for these specimens. Rerun by Trinity *ultra*² affinity method and report the *ultra*² HbA1c result. Refer to *ultra*² HPLC SOP.
 - x. Samples with heterozygous HbC will exhibit an additional peak or peaks after the A0 peak, and the instrument will designate the main peak HV-2, and minor peaks Pxx. The

chromatogram is labeled with “HbAC”, and the %HbA1c cannot be reported for these specimens. Rerun by Trinity *ultra*² affinity method and report the *ultra*² HbA1c result. Refer to *ultra*² HPLC SOP.

- xi. Samples with LA1c results $\geq 5\%$ and/or LA1c results $>$ half SA1c could indicate a possible uncommon hemoglobin variant.¹ Rerun by the Trinity *ultra*² method.
 - xii. The manufacturer recommends HbA1c results reportable with HbF less than 15%. However, data show that elevated fetal hemoglobin (HbF) up to 30.6% does not interfere with the Tosoh G8 HbA1c result as long as there is adequate separation of the HbF and LA1c peaks. This has been demonstrated based on the comparison of Tosoh G8 and the IFCC HbA1c Capillary Electrophoresis Reference Method.⁸ In the event that the LA1c peak is greater than 10% (an indication of inadequate separation of HbF from LA1c), the analysis is repeated. If separation is still not adequate, this is reported to the supervisor and the result is not reported. The following equation is then used for the calculation of a reportable result: $\% \text{ SA1c} = (((\text{SA1c Area} / (\text{A1a Area} + \text{A1b Area} + \text{SA1c Area} + \text{A0 Area})) \times 100) \times \text{Slope} + \text{Y Intercept})$.
 - xiii. Refer to the Appendix section for chromatogram printout explanation.
 - xiv. Any other abnormal chromatograms should be reported to the supervisor for further investigation prior to reporting.
- h. Result reports are generated by the G8 Reporting Software by highlighting all the specimens in the run and selecting the “Result Report x 6” function. A Result List is also generated by again highlighting all of the specimens from the run and selecting the “Result List – Portrait” function.

(6) Reporting Results:

(1) Procedure:

- a. In G8 Data Management software, select all specimens, calibrators, and controls for the run, and press the report button.
- b. Select “Report x6.” Chromotography report will open in Adobe Reader. Print.
- c. Repeat step 1 above, select Result list (portrait). Result list will open in Adobe Acrobat Reader. Print to local printer.
- d. Specimens not able to be verified under Worklist verify can be verified using Accession Result Entry application.
- e. Review the printed sheets to verify that controls meet acceptance criteria and that all specimen results requiring further verification have been noted and had their barcodes appended with the letter V to prevent inadvertent uploading of

these results. Make sure all specimens have barcodes. Then, highlight results to send to Cerner Pathnet and click "Upload" button.

- f. Open MyApps in Internet Explorer and double-click on Cerner.
- g. Log In to Pathnet Appbar.
- h. Open Worklist Request. Click on New Worklist.
- i. In the window "New Worklist," scroll down to UH HbA1c Tosoh and highlight this by clicking on it.
- j. Click OK button.
- k. List will populate with all specimens received without verified results.
- l. Using the last four buttons on the tool bar, order the list identical to the running order.
- m. Click on the "Assign Worklist ID" button in the tool bar.
- n. In "Assign Worklist ID" window, the default is <Autoassign>. Press OK Button.
- o. Close Worklist app and reopen. Open worklist created above and click print.
- p. In print window, uncheck landscape and click OK.
- q. Submit Worklist, chromatography, and result list printouts along with completed diary sheet to supervisor for verification.
- r. Supervisor will verify acceptability of entire run based upon controls, and acceptability of individual results based upon chromatography.
- s. When satisfied, the supervisor will use Cerner Pathnet Worklist Verify application to pull up worklist and verify.
- t. Estimated Average Glucose (EAG) will be calculated, performed, and autoverified by Cerner Pathnet upon verification of the HbA1c result using the below formula: $eAG = 28.7 \times HbA1c - 46.7$.⁹

(7) Panic Results:

As this test is utilized strictly as measure of long-term glycemic control, there are no "panic values" for this test and therefore this section is not applicable.

(8) Reporting Format:

Results are expressed on the report as % Hemoglobin A1c (HbA1c) and are rounded to one decimal place.

Results are reported throughout the entire range of % HbA1c values verified by linearity studies. Results below 4.0% or above 14.0% are reanalyzed for verification prior to results being reported. Results outside of current linearity values are reported as < (low linearity value) or > (high linearity value) as appropriate. The latest linearity data is found in the Environmental Control and Instrument Maintenance binder under the

Linearity tab. Refer to the linearity procedure in the Clinical SOP binder for up-to-date linearity values. As of 12 February 2013, the limits were 3.1 – 19.5%. Linearity studies are performed every 6 months.

(9) Supervisor Responsibility:

The supervisor ensures quality control passes within the acceptable ranges prior to releasing patient results.

The supervisor checks every individual chromatogram to ensure all peaks are resolved and reportable.

All chromatograms requiring further evaluation are noted and are has not uploaded into pathnet.

The Result Report is checked against the Laboratory Worksheet

(10) Procedure Notes

- (1) To avoid an error condition during calibration, be sure to place PLC5 and PHC5 in the first sample rack in positions 1 and 2 respectively.
- (2) Each reagent lot number supplied by Tosoh is performance matched to the supplied TSKgel G8 Hsi Columns. Following any announced change in supplied Tosoh TSKgel G8 HSi Columns, contact Tosoh to determine suitability of existing reagents.
- (3) The reagents must be at room temperature prior to use.
- (4) If the column is not to be used for more than one week, remove it from the analyzer, seal the ends with the protective plugs and store in cool place at 4-15 °C. Avoid direct sunlight.
- (5) The relationship between HbA1c results from NGSP network and the IFCC network is expressed by using the following equation: $NGSP (\%) = 0.09148 \times IFCC (mmol/mol) + 2.1527$
- (6) Any changes to procedure must be documented. Major changes to the SOP may include the way a procedure is performed or calculations and requires the approval of the Medical Director. Minor changes include typographical errors or other minor corrections that do not change the way the procedure or calculation is performed and do not require approval of the laboratory director. Major SOP changes must be reviewed by the Lab Director prior to SOP update.
- (7) Any changes to the SOP will be communicated to technical staff via verbal communication and email notification. Technical staff after reading the changes made to the SOP will review, sign, and date the SOP.

9. REPORTABLE RANGE OF RESULTS

Mean	5.0%
Range	4 - 6 % (equivalent of mean blood glucose of 60 - 120)

mg/dL)

The normal range for the HbA1c test was established at the Diabetes Diagnostic Laboratory in February 2000 based on 181 non-diabetic subjects collected from the continental United States. Subjects were confirmed to have fasting blood glucose less than 110 mg/dL (2000 standard for non-diabetic classification). The mean HbA1c was 5.0%, with a 99% Confidence Interval of 4 to 6%. Reference Range studies were repeated in 2004, 2009 and 2012 in the same manner except that subjects were included only if their fasting glucose was less than 100 mg/dL (Current ADA criteria for non-diabetic classification). In all studies the original range of 4-6% was confirmed.

10. QUALITY CONTROL (QC) PROCEDURES

(1) “Bench” quality control specimens.

a. Control Materials and stability

- i. Six 7ml K₂ EDTA Vacutainer tubes of venous whole blood were drawn from known non-diabetic individuals (Normal level HbA1c). The elevated (Abnormal) HbA1c level whole blood (EDTA) controls were purchased from Aalto Scientific. Refer to Aalto Scientific product whole blood quality assurance report for additional details.
- ii. For the normal level, the tubes were pooled, 50 µL aliquots were dispensed into 400µL microtubes at 4°C, and stored at -70°C. The remaining aliquots were placed in a cryogenic (liquid nitrogen) tank at -196°C.
- iii. Controls are stable for at least two years at -70°C, and up to five years or longer in liquid nitrogen. When controls are transferred from liquid nitrogen to -70°C, an expiration date is written inside the lid of the box. transferred that is two years from the transfer date.
- iv. Refer to specimen section for stability requirements.

b. Preparation for Analysis

Three 1.5mL sample vials of each level are prepared in the same manner as an unknown specimen as described in PROCEDURE—STEPWISE section 4.

c. Mean and Ranges

- i. Daily means and ranges were calculated from twenty interassay determinations.
- ii. Quality control limits are established by calculating 95% (2sd) and 99% (3sd) confidence limits for both daily means and daily ranges for each control.

- iii. Mean and Range limits for the current controls are posted on each G8 instruments.*

***Current Control value assignments and limits can be found in the Quality Control Binder located in room M771.**

d. Tolerance Limits

1. Analytical Batch Quality Control - Daily

The system is declared “out of control” if any of the following conditions occur:

- a. The mean from a single run for a single control falls outside 99% confidence limits (3sd).
- b. The means from a single run for both controls fall outside 95% confidence limits (2sd).
- c. The means from eight successive runs for a single control fall either all above or all below the mean line. Runs for which the mean falls within 1sd of the established mean are not counted in this trend.
- d. The range from a single run for a single control falls above 99% confidence limits.
- e. The ranges from a single run for both controls fall above 95% confidence limits.
- f. The ranges from eight successive runs for a single control fall above the mean line.

2. If a run is declared “out of control”, all patient samples from that runs are repeated in another run. Additionally, the instrument, calibration, and controls are investigated to determine the cause of the problem before further analysis occurs. In the case of a trend, troubleshoot accordingly by either adjusting the flow rate and/or performing a recalibration. Refer to the G8 Variant Analysis Mode Training Manual (pg 47) or the G8 Operator’s Manual (chapter 6) for additional Troubleshooting guidelines. ¹

e. Levey-Jennings Plots - Monthly

1. Mean chart—Plots the mean values for each control run and each compares them to upper and lower two and three standard deviation limits as well as the mean.
2. Range chart— Plots the range values (maximum value – minimum value) for each control in each run and compares them to mean, upper two standard deviation, and upper three standard deviation limits.
3. Charts are generated after each analytical batch and reviewed by the supervisor prior to validating result.
4. The Laboratory Director reviews these on a monthly basis.

(2) Routine Controls Testing

- a. Normal and elevated controls are run at the beginning and at the end of a run, AND
- b. Controls are run every 19 samples, alternating between normal and elevated levels.

(3) Sample QC

- a. Five percent of specimens are randomly selected and reanalyzed in another run.
If the difference in %HbA1c between the duplicate is greater than 10% (relative) of the original HbA1c value, the specimen is again reanalyzed and the chromatograms, instrument, and QC data from both the original and duplicate runs are investigated. The duplicate results are entered in a database weekly and reviewed weekly by a supervisor.

(4) Inter-instrument QC

- a. Comparison between Tosoh G8 instruments
NGSP monitoring specimens are analyzed each month on each Tosoh G8 HPLC instrument to validate agreement between instruments. Acceptability is defined as Chi-squared (χ^2) = 0.2290 control limit (99 %), warning limit of 0.2030 (95%) at $\alpha=0.01$ and DF=10.
- b. Comparison between Tosoh G8 and Trinity Primus *ultra*² instruments
 - i. GHBQC procedure (external) is followed for the comparison of results
(n = 30 – 50 per month) between the Tosoh G8 HPLC HbA1c method and its backup method, Trinity Primus *ultra*² HPLC HbA1c. Criteria for Pass/Fail: Bland/Altman: +/-0.70, Y at X: +/-0.30, Syx: 0.35
 - ii. Results are reviewed monthly by the Lab Director.

(5) Proficiency Testing

- a. College of American Pathologists (CAP) survey.
- b. Twice yearly DDL will receive Proficiency Testing (PT) specimens from CAP.
 - i. One package is to be used for Tosoh G8 HbA1c PT.
 - ii. One package is to be used for Trinity Primus *ultra*² PT.
- c. PT samples are tested as patient samples within the routine lab workload.
- d. PT results are recorded on the forms included in the shipment, other fields in the form are completed, and then submitted to CAP online by the lab supervisor.
- e. CAP will mail a summary report showing how the lab performed compared to other labs/methods and the assigned target values.

- f. The Lab director will sign and review the attestation form from the PT result.
- g. After review, results and form are filed in the Tosoh G8 Proficiency Testing binder stored in M771.

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

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

For diagnostic purposes, the results obtained from this assay should be used in conjunction with other data (for example, signs and symptoms, duration of diabetes, results of other test, age of patient, clinical impressions, degree of adherence to therapy, etc.).

The life span of red blood cells is shortened in patients with hemolytic anemias, depending upon the severity of the anemia. As a consequence, specimens from such patients may exhibit decreased glycohemoglobin levels.¹

The life span of red blood cells is lengthened in polycythemia or post splenectomy patients. Specimens from such patients may exhibit increased glycohemoglobin levels.¹

Interfering Substances:

The presence of hemoglobin variants (e.g. HbC, HbF >30.6%, HbE, HbD, etc.) may interfere with HbA1c results.⁵

Below shows no interference^{1, 5}:

1. Labile A1c as indicated by glucose concentrations ≤ 1000 mg/dL or La1c < 5.0% of the total area
2. Icterus ≤ 20 mg/dL
3. Lipemia ≤ 1000 mg/dL
4. Sodium cyanate ≤ 25 mg/dL
5. Alcohol ≤ 25 mg/dL
6. Aspirin ≤ 50 mg/dL

13. REFERENCE RANGES (NORMAL VALUES)

Range 4 - 6 % (equivalent of mean blood glucose of 60 - 120 mg/dL)
Reference Range studies were repeated in 2004, 2009 and 2012 in the same manner except that subjects were included only if their fasting glucose was less than 100 mg/dL (Current ADA criteria for non-diabetic classification). In all studies the original range of 4-6% was confirmed.

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; PROTOCOL FOR REPORTING CRITICAL CALLS (IF APPLICABLE)

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

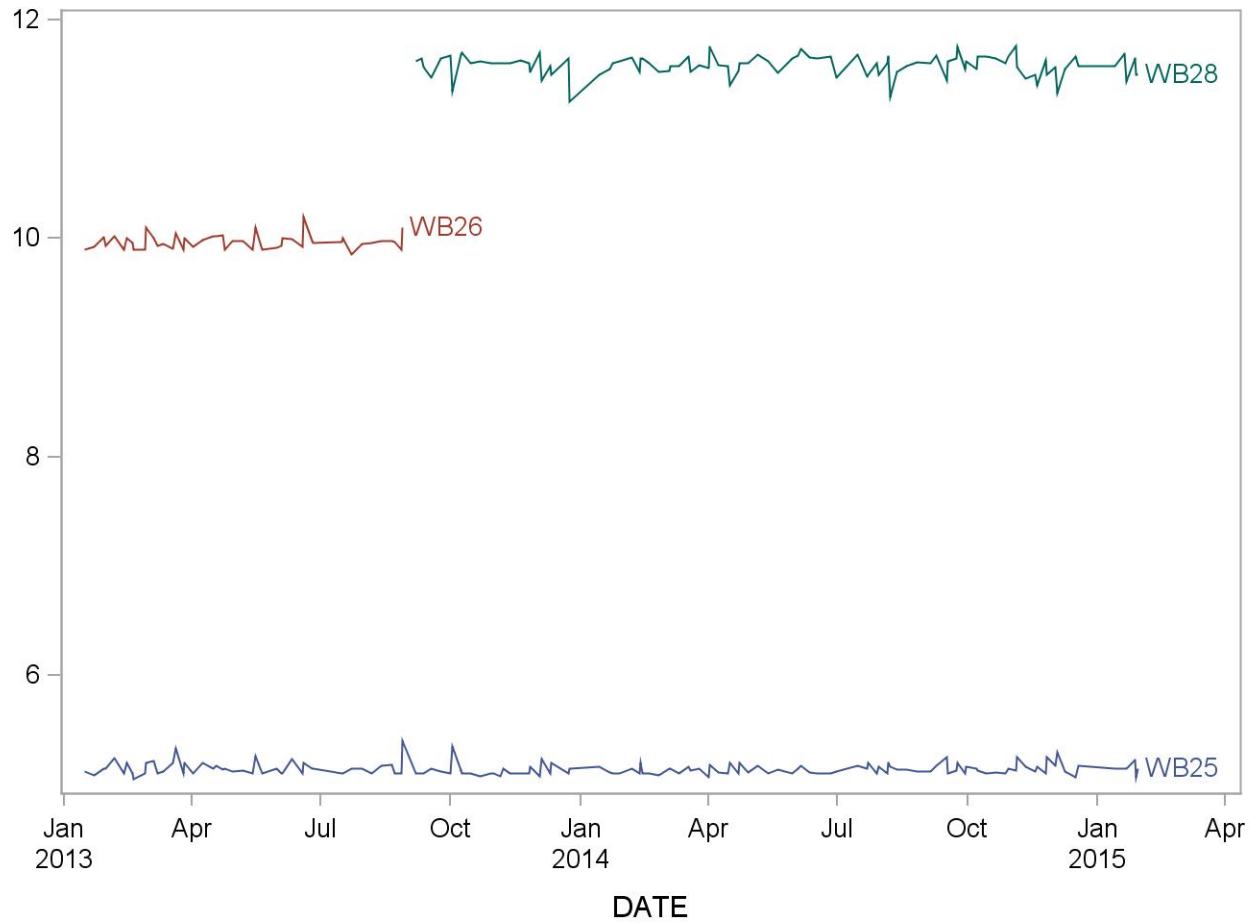
All shipments are recorded on the NHANES Shipping Log upon receipt. Actions taken during the course of analysis, result reporting, and specimen retention are also recorded on the log.

19. SUMMARY STATISTICS AND QC GRAPHS

See following pages.

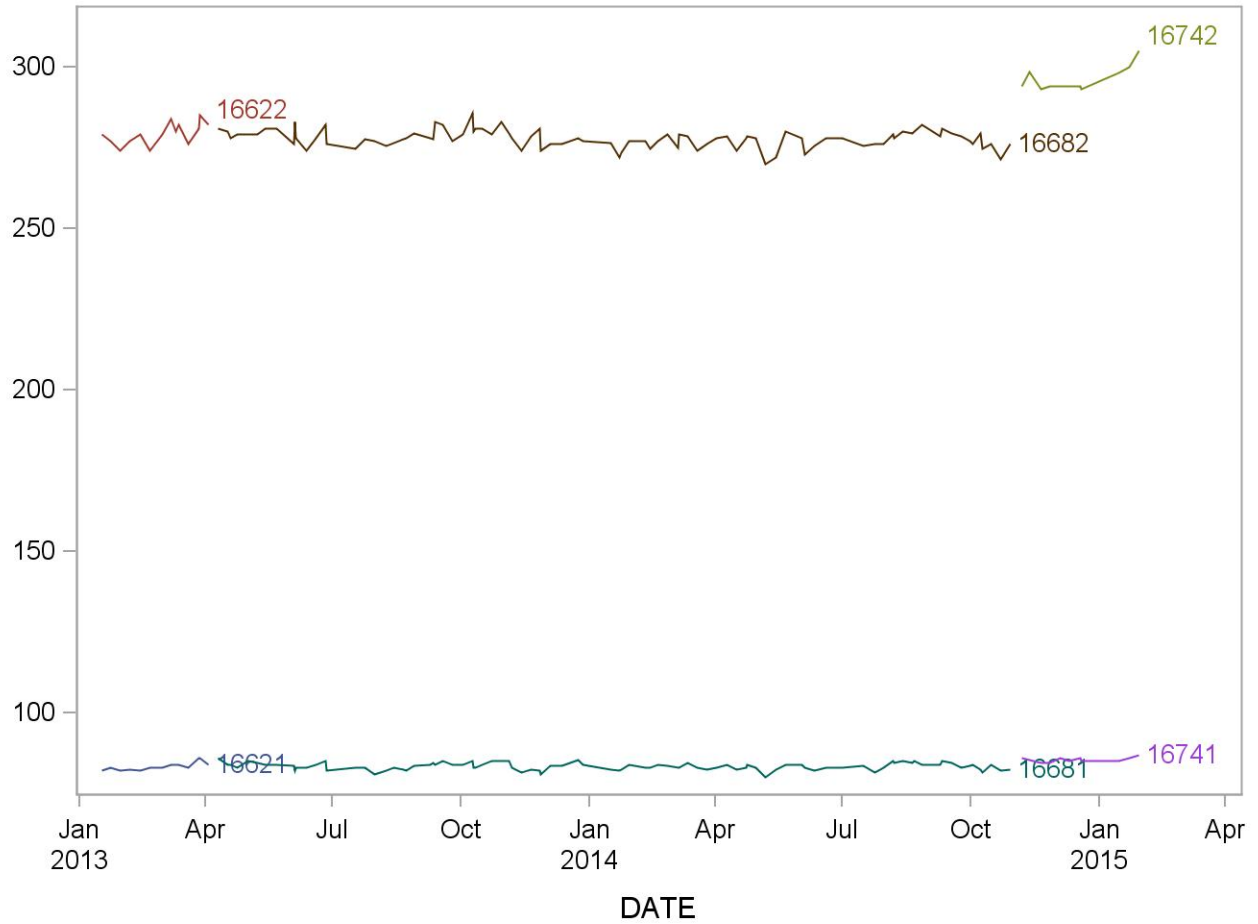
2013-2014 Summary Statistics and QC Chart for Glycohemoglobin (%)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
WB26	38	15JAN13	27AUG13	9.9178	0.0363	0.4
WB25	118	15JAN13	28JAN15	5.1018	0.0183	0.4
WB26	33	22JAN13	28AUG13	10.0298	0.0828	0.8
WB25	96	22JAN13	29JAN15	5.2002	0.0665	1.3
WB28	80	06SEP13	28JAN15	11.6298	0.0565	0.5
WB28	64	12SEP13	29JAN15	11.5306	0.1263	1.1



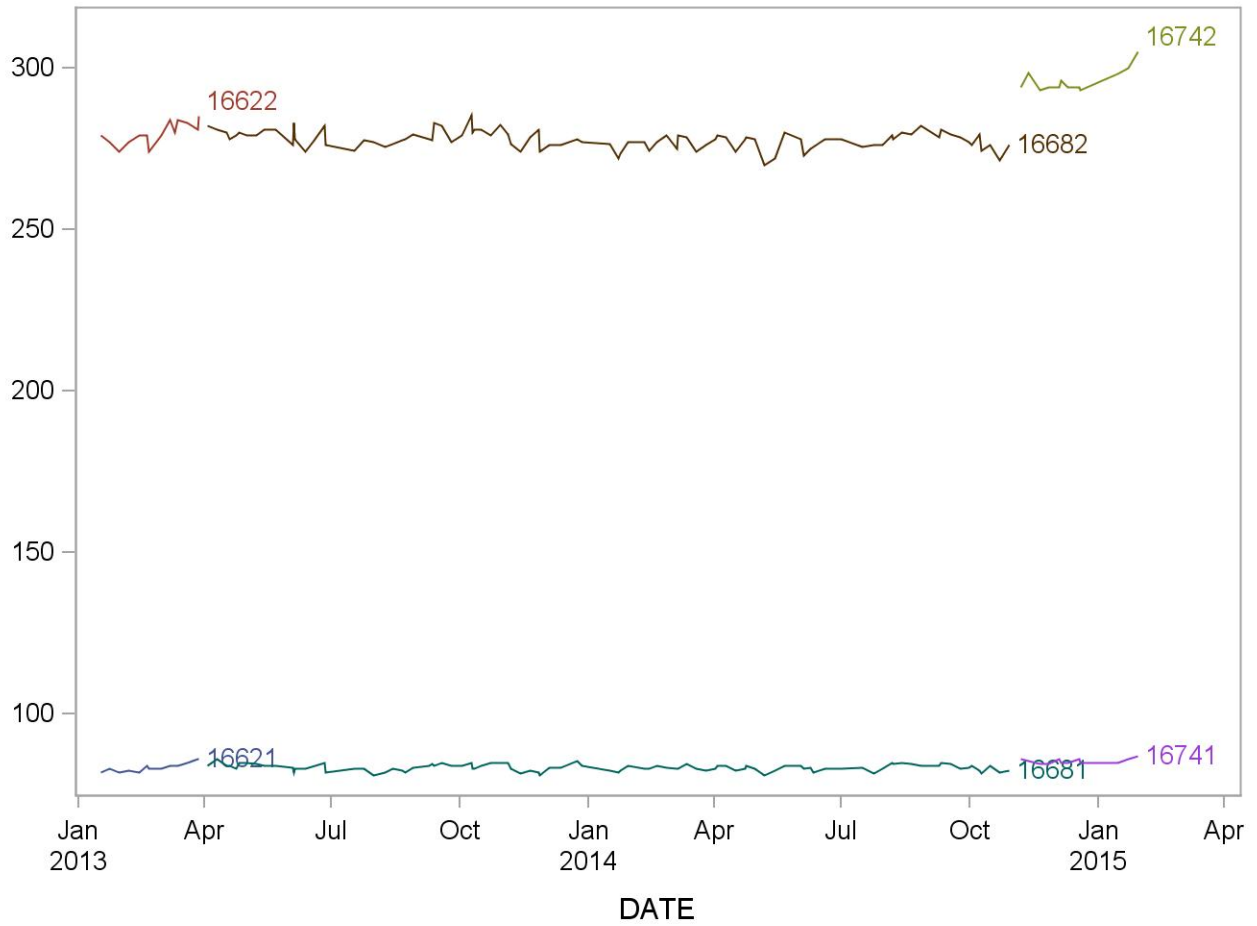
2013-2014 Summary Statistics and QC Chart for Two hour oral glucose tolerance (OGTT)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
16622	13	17JAN13	28MAR13	279.0	3.4	1.2
16621	13	17JAN13	28MAR13	83.4	1.4	1.6
16682	64	10APR13	30APR14	278.0	2.7	1.0
16681	64	10APR13	30APR14	83.4	1.1	1.3
16682	28	07MAY14	29OCT14	277.1	3.0	1.1
16681	28	07MAY14	29OCT14	83.4	1.2	1.5
16742	11	06NOV14	29JAN15	296.1	3.8	1.3
16741	11	06NOV14	29JAN15	85.5	0.8	0.9



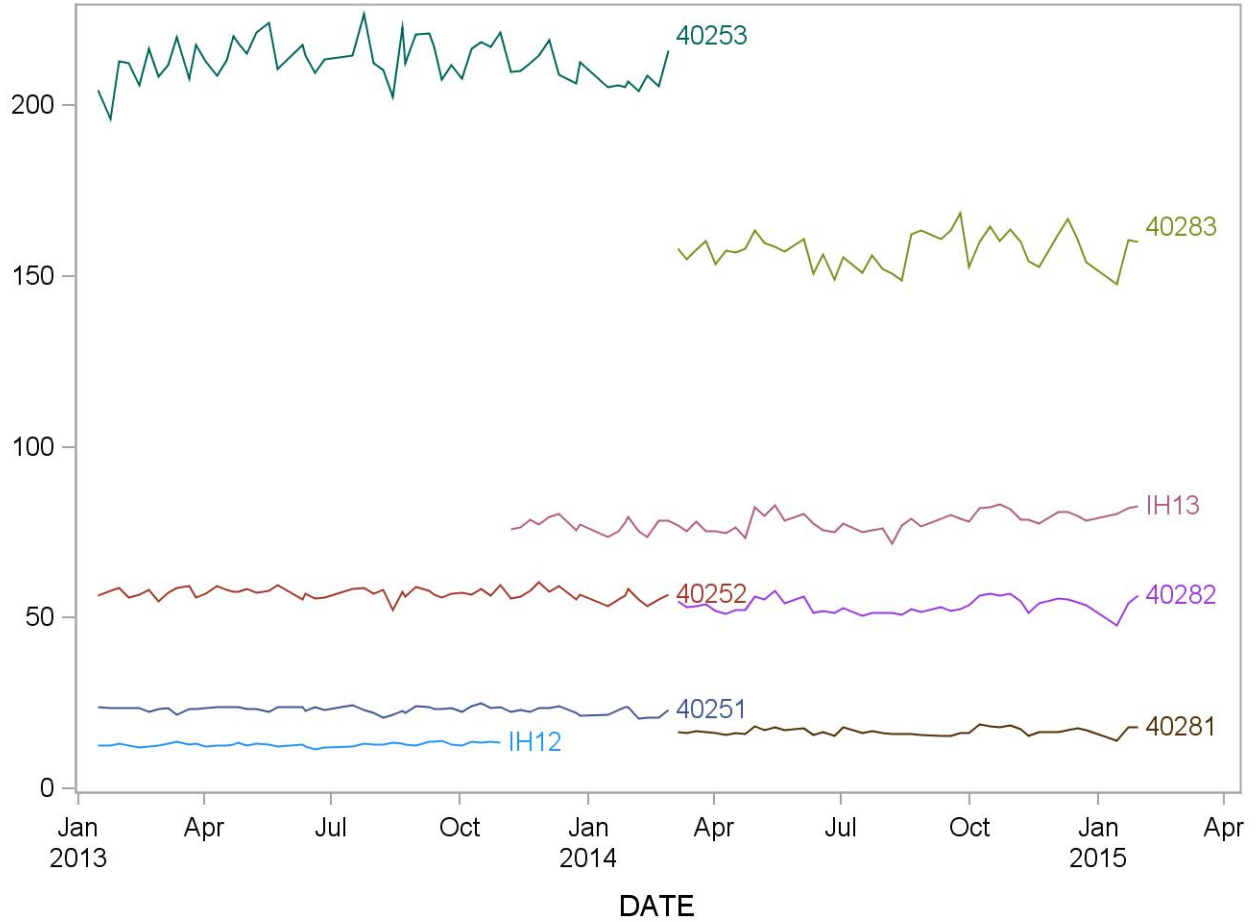
2013-2014 Summary Statistics and QC Chart for Plasma Glucose (mg/dL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
16621	14	17JAN13	28MAR13	83.6	1.4	1.6
16622	14	17JAN13	28MAR13	279.6	3.5	1.3
16681	49	03APR13	30APR14	83.5	1.1	1.4
16682	49	03APR13	30APR14	278.7	2.7	1.0
16681	27	30OCT13	09APR14	83.3	1.1	1.3
16682	27	30OCT13	09APR14	276.9	2.4	0.9
16681	28	07MAY14	29OCT14	83.3	1.1	1.3
16682	28	07MAY14	29OCT14	276.9	2.9	1.1
16741	12	06NOV14	29JAN15	85.5	0.8	0.9
16742	12	06NOV14	29JAN15	296.1	3.6	1.2



2013-2014 Summary Statistics and QC Chart for Insulin (uU/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
40253	57	15JAN13	27FEB14	212.8375	6.0921	2.9
40251	57	15JAN13	27FEB14	23.1567	1.0138	4.4
40252	57	15JAN13	27FEB14	57.2089	1.6298	2.8
IH12	41	15JAN13	30OCT13	12.9979	0.5393	4.1
IH13	58	06NOV13	29JAN15	78.1380	2.6873	3.4
40283	42	06MAR14	29JAN15	157.8612	4.9979	3.2
40281	42	06MAR14	29JAN15	16.8162	1.0216	6.1
40282	42	06MAR14	29JAN15	53.6002	2.2203	4.1



REFERENCES

- (1) National Glycohemoglobin Standardization Program (NGSP) website:
[http:// www.ngsp.org](http://www.ngsp.org)
- (2) Tosoh Automated Glycohemoglobin Analyzer HLC-723G8 Variant Analysis Mode Operator's Manual v. 3.0 , Tosoh Bioscience, Inc. 2014 and Training Manual.
- (3) *Ultra²* SOP.
- (4) Validation Study Binder for HbA1C.
- (5) Effects of whole blood storage on hemoglobin a1c measurements with five current assay methods. Rohlfing CL, Hanson S, Tennill AL, Little RR. *Diabetes Technol Ther.* 2012 Mar;14(3):271-5. doi: 10.1089/dia.2011.0136. Epub 2011 Oct 27
- (6) Effects of Sample Storage Conditions on Glycated Hemoglobin Measurement: Evaluation of Five Different High Performance Liquid Chromatography Methods. *Diabetes Technol Ther.* 2007 Feb;9(1):36-42. Little RR¹, Rohlfing CL, Tennill AL, Connolly S, Hanson S.
- (7) Stability of whole blood at -70°C for measurement of hemoglobin A1c in healthy individuals. *Clin Chem* 2004;50:2460-2461 Jones W, Scott J, Leary S, Stratton F, Jones R; ALSPAC Study Team.
- (8) Tosoh G8 Variant Analysis Mode Chromatogram Interpretative Guide.
- (9) Verbal Communication - Tosoh Bioscience Field Service Engineer, Mark Scheckel
Tel: (650)-636-8350
Email: mark.scheckel@tosoh.com
- (10) National Glycohemoglobin Standardization Program (NGSP) website:
[http:// www.ngsp.org](http://www.ngsp.org)
- (11) Little RR, Rohlfing CL, Hanson SE, Schmidt RL, Lin CN, Madsen RW, Roberts WL. "The Effect of Increased Fetal Hemoglobin on 7 Common Hb A1c Assay Methods." *Clin Chem.* 2012 May 1; 58(5): p. 945.
- (12) Nathan DM, et al. Translating the A1c Assay into Estimated Average Glucose Values. *Diabetes Care* 31:1473-8, 2008