

# Laboratory Procedure Manual

*Analyte:* **Glycohemoglobin (HbA1c)**

*Matrix:* **Whole Blood**

*Method:* **Tosoh G8 High Performance Liquid Chromatography (HPLC) Glycohemoglobin Analyzer**

*Revised Date:* **May 5, 2020**

*As performed by:* **Diabetes Diagnostic Laboratory  
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## **Important Information for Users**

The Diabetes Diagnostic Laboratory 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:

<b>Data File Name</b>	<b>Variable Name</b>	<b>SAS Label</b>
GHB_K	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.<sup>1</sup> This test is also used as an aid in the diagnosis of diabetes identifying patients who may be at risk for developing diabetes.<sup>2</sup>

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 ~ 120 days).

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

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 a deterioration peak, an abnormal looking chromatogram, or a presumptive hemoglobin variant are reflexed to the boronate affinity method. In addition, G8 results  $\leq 10\%$  HbA1C that have a LA1C  $\geq 1/2$  SA1C are reflexed to the boronate affinity HPLC method for verification.

## **2. SAFETY PRECAUTIONS**

1. While working in the lab proper Personal Protective Equipment (PPE) is enforced. This includes wearing gloves, lab coats, face masks (as applicable), protective eye wear such as goggles, and closed toe shoes are required for handling all human blood specimens. Once gloves are removed wash your hands or use hand sanitizer to ensure your hands are clean before leaving the lab.
2. Vials containing human blood are only to be opened in a biological safety cabinet with the sash in the correct position.
3. All plastic tips, sample cups, gloves, etc. that contact blood are considered contaminated and are to be placed in a biohazard waste container.
4. 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. Refer to the Lab Safety Manual for additional details located in room M764.
5. 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.

## **3. COMPUTERIZATION; DATA SYSTEM MANAGEMENT**

Data are maintained on a secured Microsoft Access / Microsoft SQL server client-server system in a 128-bit authenticated Windows domain environment.

1. Laboratory services are requested through the Westat system operations via an email notification containing a unique manifest list of the samples and sample analysis type (e.g. GHB), which confirms that specimens have been shipped to DDL.
2. Each Manifest Form should include and be verified against each sample received:
  - a. Patient Sample ID #

- b. Test Name
  - c. Date Collected
  - d. Shipment ID #
  - e. Shipment Date
  - f. Lab Name
  - g. Lab ID
  - h. Survey Year
3. Once specimens are received and verified the corresponding file is imported electronically into the SQL server database via secure transfer.
  4. After analysis the results, date analyzed and tech initials are imported from the instrument into the SQL server database via secure transfer.
  5. Data check sheets are printed out and checked against the instrument printouts by the supervisor.
  6. After results are cleared by the supervisor a results file in the specified format is exported and uploaded to Westat via secure transfer.

#### **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. Specimen Type and stability:
  - a. Collect whole blood specimens in vacuum collection tubes containing EDTA anticoagulant (purple or pink tops) and mix thoroughly.
  - b. As per manufacturer 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.<sup>1</sup> However, a stability performed in-house and published through Diabetes Technology & Therapeutics demonstrated the below WB sample stability<sup>4-7</sup>
    - i. Room Temperature: 6 days
    - ii. 4°C: 14 days
    - iii. Frozen (- 20°C);(-70°C): 7 days; 1 year.
  - c. HbA1c samples are stable at frozen conditions of – 70 °C for 10 years (refer to the Stability of Haemoglobin A1c (HbA1c) measurements from frozen whole blood samples stored for over a decade study; 2005 Diabetes UK, Diabetic Medicine, 22, 1726 – 1730).
  - d. Liquid Nitrogen for up to 10 years
  - e. 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.<sup>1</sup>
  - f. Specimens collected using the Bio Rad HbA1c Capillary Collection System are also acceptable for research testing use only.<sup>8</sup> Samples prepared using this procedure are stable for 2 weeks at room temperature or 4 weeks at 2-8 °C or 4 days at 42°C.

3. Specimens are delivered to the Diabetes Diagnostic Laboratory, Room M764 by overnight courier. Each specimen must arrive in the laboratory with a unique barcode identification number. Unacceptable specimen criteria:
  - a. Clotted samples
  - b. Specimen types and stability not listed above.
  - c. Unlabeled samples.
4. If an unacceptable specimen is received notify NHANES and Westat, and add the appropriate comment code in the database.
5. Handling Conditions:
  - a. Samples are to be kept refrigerated at 2-8°C immediately after collection.
  - b. Transport under refrigerated conditions.
  - c. Once received and prepared for analysis, specimens are to be immediately returned to 2-8°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).<sup>7</sup>

## 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).

<u>Parameter</u>	<u>Setting</u>
Wavelength	Sample: 415nm Reference: 500nm
Column Temperature	25 °C
Injection Interval	1.6 min
Calibration	Two-point
Working Temperature	15°C - 27°C

- b. Hamilton Autodilutor Model Microlab 500 or 600 with 2.5 mL and 25  $\mu$ L 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. Adjustable Variable Volume Pipettes in 0.5-10, 2-20, 20-200, and 100-1000  $\mu$ L volumes.
2. Equipment Maintenance
- a. Tosoh Analyzer System—Routine maintenance
    - i. 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.<sup>1</sup> At least 5 prefilters should be on hand at all times.
    - ii. Analytical column—Change after 2500 injections.<sup>1</sup> At least two spare columns are to be kept available at all times.
    - iii. Record all routine maintenance in the Tosoh G8 Diary.
  - b. Tosoh Analyzer System—Periodic/Preventative Maintenance
    - i. Replacing Buffers: Solution of Hemolysis Wash
    - ii. 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.
    - iii. 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.
    - iv. Write the date opened and an expiration date three months into the future on each bag or bottle as it is opened.
    - v. From the MAIN screen, select MAINTE, then REAGENT CHANGE. Once the buffers have been correctly installed, select the appropriate buffer, and then press CHANGE.
    - vi. Record new lot information in G8 Data Management software, daily diary sheet, and buffer record in G8 Diary sheet binder.
  - c. Removing air from the buffer lines
    - i. 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:
    - ii. From the MAINTE screen, press REAGENT CHANGE key.
    - iii. Highlight the key(s) for the reagent(s) to be primed.
    - iv. 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.
    - v. Pump buffers and verify pressure. Repeat if necessary.
  - d. Removing air from the pump

- i. During pumping, if the pressure does not rise, air may be present on the outlet side of the pump.
  - ii. Use the following procedure to remove the air.
  - iii. Verify analyzer is in STAND-BY mode.
  - iv. If the analyzer is not in STAND-BY mode, press the STOP key and wait until 'STAND-BY' appears on the Status screen.
  - v. Press the REAGENT CHANGE key on the MAINT screen.
  - vi. Press the DRAIN FLUSH key.
  - vii. 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."
  - viii. Turn the valve ONLY 90 degrees counterclockwise.
  - ix. Press the OK key.
  - x. A confirmation message will appear. Ensure the drain valve is open. Press the OK key again.
  - xi. 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.
  - xii. 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.
  - xiii. Press the OK key.
  - xiv. Press the EXIT key to return to the main screen second page. Press the PUMP key.
  - xv. 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.
- e. Replacing the Filter Element
- i. Replace the filter element after 400 injections or when the pressure exceeds limits as indicated on the column inspection report +4MPa.
  - ii. Verify analyzer is in STAND-BY mode.
  - iii. If the analyzer is not in STAND-BY, press the STOP key and wait until 'STAND-BY' appears on the Status screen.
  - iv. Open the door below the display.
  - v. Confirm that the SV1 key is open (O) on the second page of the main screen.
  - vi. Remove the filter outlet (peek) tubing from the top of the filter assembly.
  - vii. Loosen the top of the filter holder assembly by turning it counterclockwise. Remove the filter holder by pulling it straight out.
  - viii. 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.
- ix. Firmly tighten the top of the filter holder assembly by hand until no further tightening is possible.
  - x. Slide outlet tubing unit it extends  $\frac{1}{4}$  inch past the end of the tubing. Connect the outlet side tubing.
  - xi. 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.
  - xii. Press the PUMP key to stop the pump.
  - xiii. Reset filter counter to 0 on the REAGENT CHANGE screen.
  - xiv. Record change on daily diary sheet.
- f. Column Replacement: Replace the column in the following situations.
- i. Replace column after 2500 injections.
  - ii. When the pressure is more than what is indicated on the column inspection report + 4 MPa and is not reduced by filter replacement.
  - iii. When peaks on the chromatogram (particular the shaded SA1c peak) have become broad or broken in two fractions.<sup>1</sup>
  - iv. When assay results for quality control samples are consistently out of assigned ranges even after re-calibration.
  - v. When the CALIB ERROR persistently occurs.
  - vi. Please contact Technical Support if the above issues are not resolved after column replacement.
  - vii. 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.
    - 1) 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 column.
    - 3) Open the front doors of the analyzer. Release latch and open the column oven. Unscrew column connections and remove used column.
    - 4) Confirm that the SV-1 key is open (O) on the MAIN screen, (second page).
    - 5) 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.
    - 6) 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.

- 7) Check for leaks. Press the PUMP key to start the pump and confirm there is no fluid leakage.
  - 8) Check for fluid leaks at the connections. If leaks occur, tighten fittings.
  - 9) 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.
  - 10) 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.
  - 11) After verifying connections are secure, stop the pump by pressing the PUMP key.
  - 12) Close column oven.
  - 13) Close front doors of the analyzer.
  - 14) After connecting a new column, reset (zero) to the column counter in the REAGENT CHANGE screen.
  - 15) Record change on daily diary sheet.
  - 16) 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.<sup>5</sup>
  - 17) If necessary, adjust the flow rate to match the retention time for the SA1c peak on the reference chromatogram included with the column.
  - 18) 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 or delegee for usage approval.
  - 19) 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:
    - a) XY plot (current lot on x-axis) with linear regression performed.
    - b) Slope = 1.0+/- 0.1
    - c) Intercept = 0.0+/- 0.1
    - d)  $R^2 > 0.98$
    - e) 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.
- g. Replacing printer paper.
- i. Lift the printer cover (upper lid) to the back to open.

- ii. Push the paper holding lever down to the very front and wrap the remaining paper onto the roll.
  - iii. Lift the roll up and remove the mandrel.
  - iv. Insert the mandrel into the new roll with attention to the direction.
  - v. Return the paper holding lever to the very back and insert the paper into the printer. Press the feed switch to feed the paper.
  - vi. 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.
- h. Replacing the sampling needle.
- i. 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.
  - ii. Put on protective clothing (goggles, gloves, etc.) and take care not to touch the end of the sampling needle during handling.
  - iii. Press the POWER key to switch off the analyzer.
  - iv. Use a screwdriver to remove the sampling cover screws.
  - v. Remove the sampling needle cover.
  - vi. 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.
  - vii. 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.
  - viii. By hand, loosen and remove the tubing connected to the 3-way block.
  - ix. 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.
  - x. Remove the screws that hold the guide through which the tubing passes.
  - xi. Slowly lift up the sampling needle to remove it. Place immediately into a sharps container.
  - xii. 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.
  - xiii. Secure the holding plate with the screws.
  - xiv. Pass the tubing through the guide, secure with the screw, and securely connect the tubing to the 3-way block.
  - xv. 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.

- xvi. 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.
  - xvii. 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.
  - xviii. Adjusting the Flow Rate: The flow factor is generally 1.00 mL/min, but can be 1.02/1.03 mL/min dependent on instrument factory setting. The flow factor should only be adjusted +/- 0.05 of the default factory setting.<sup>1</sup>
3. Instrument Preventative Maintenance (PM)
- a. A monthly PM is performed by a trained member of our DDL staff using the monthly PM checklist (located on the H:\DDL\D150\Templates drive). The completed checklist is reviewed by the lab supervisor or delegee for verification. The Monthly PM is performed using the below steps;
    - i. With G8 powered on, press the menu button, then utilities, then password.
    - ii. Enter "MAINTE" in password screen, then press enter.
    - iii. Exit back to main screen.
    - iv. Press Mainte button.
    - v. Press Sampler Mech button.
    - vi. Remove the fitting from the top of the filter holder assembly.
    - vii. Remove the top portion of the filter assembly.
    - viii. Eject the filter.
    - ix. Wet a cotton swab with DI water. Clean inside of filter holder assembly, including the threads. Clean the top portion of the assembly as well.
    - x. Reassemble the filter assembly.
    - xi. Remove the two screws from the top of the blue cover door, and pull open cover.
    - xii. Press the "Move Y stat" button. The needle should move to the stat position.
    - xiii. Remove the two screws holding down the needle. Remove the small metal plate under the screws.
    - xiv. Remove the needle.
    - xv. Remove the screws from the bottom portion of the needle assembly. Remove the metal plate beneath these screws.
    - xvi. Remove the blue o-ring.
    - xvii. Clean the screws, plates, and o-ring.
    - xviii. Using an alcohol wipe, clean the needle to remove any dried blood. Use the corners of the alcohol wipe's package to clean out the grooves on two sides of the needle.
    - xix. Use the alcohol wipe to clean the positioning wheel behind the stat well.
    - xx. Replace the o-ring. Place one drop of TriFlow lubricant in the middle of the o-ring.

- xxi. Replace the metal plate that holds the o-ring in place, and replace the screws.
  - xxii. Insert the needle into the top hole, followed by the lower hole. Assure that the bevel of the needle tip is facing you.
  - xxiii. While holding needle in place, replace the metal plate at the top, and screw it into place.
  - xxiv. Assure needle is secure and press “Move Y Dil” button.
  - xxv. Use compressed air to blow out dust from interior, metal facing under blue cover, under the printer cover, and over any vents and fans on the exterior.
  - xxvi. Replace blue cover and screw into place.
  - xxvii. Using Oxivir, clean all exterior surfaces.
  - xxviii. Remove buffers from chrome holder, remove chrome holder.
  - xxix. Rinse chrome holder under tap water, dry with paper towel, and replace holder and buffers.
  - xxx. Clean sysmex racks with Oxivir, rinse, and replace broken adapter rings.
- b. For every 20K injections, a PM is performed by a Tosoh Service representative.<sup>10</sup>
4. Pipette Preventative Maintenance
- a. Hamilton Autodilutor 500/600
    - i. Verify calibration of the device according to Autodilutor SOP.
    - ii. Instrument should be cleaned with disinfectant and inspected for proper functioning daily.
  - b. Adjustable volume pipettes
    - i. After each use, the pipette should be wiped with disinfect with soaked gauze.
    - ii. Pipettes are calibrated annually by trained field service personnel.
5. Materials:
- a. Reagents—Supplied by Tosoh Bioscience (South San Francisco, CA). Part numbers are subjected to change. Any questions or concerns about the materials used for this assay please refer to your supervisor/delegee or call Tosoh Scientific hotline at 1-800-248-6764, customer # 1208. Refer to Operator’s Manual for additional details relating to the Tosoh G8 HPLC instrument components and items used for testing.
  - b. Other Materials
    - i. Powder free nitrile exam gloves. (Fisher Scientific, Waltham, MA)
    - ii. Oxivir disinfectant. (AHP Technology, Sturtevant, WI)
    - iii. Gauze Sponges 4x4 not sterilized (Fisher Scientific, Waltham, MA)
    - iv. Kim Wipe lintless tissues. (Fisher Scientific, Waltham, MA).
6. 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 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.
- e. Reagent labeling: Reagents, calibrators, controls, and solutions should be traceably identified to indicate the following:
  - i. Content and concentration
  - ii. Storage requirements (see above stability requirements for whole blood).
  - iii. The below should be followed for working reagents; (open reagents in use for testing):
    - 1) Preparation date or opened date and the identity of the preparer.
    - 2) Tech's initials.
    - 3) Expiration date.
  - iv. Controls and calibrators obtained from freezer for daily use should be initialed and dated with the pulled date.
  - v. Prior to processing controls and calibrators, sample cups should be labeled (with the name of the control or calibrator), dated, and initialed (tech responsible for adding hemolysis wash to the sample cups).
  - vi. Processed controls and calibrators are immediately returned to the refrigerator after samples are assayed from the morning run, and are discarded after daily use.
  - vii. Buffers and wash should be labeled with the name of the reagent and reagent source (name of vendor e.g. Tosoh), preparation date or opened date, expiration date (from open or preparation date), and tech's initials.

## **7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES**

- 1. Calibrator Preparation: Donors are recruited and compensated for their donation of blood. K<sub>2</sub>EDTA whole blood tubes are pooled together, mixed for at least 30 min, and aliquoted under refrigerated conditions, see below.
  - a. Pooled Low Calibrator
    - i. Single level calibrator prepared from K<sub>2</sub>EDTA whole blood drawn by venipuncture from four non-diabetic individuals.
    - ii. The blood specimens were pooled, dispensed in 30uL aliquots into 400µL microtubes under refrigerated conditions. Batches of these low calibrators (aliquots) were assigned a lot number, preparation date and stored at -70°C on the same day. The remaining aliquots

- were assigned the same lot number/date and placed in a cryogenic (liquid nitrogen) tank at  $-196^{\circ}\text{C}$  in freezer boxes in the same day.<sup>5-7</sup>
- iii. Low calibrator HbA1c values were assigned by twenty interassay determinations along with the previous lot of calibrator. Refer to QC and Calibrator's binder and the controls and calibrator color key table (locate at the bench) for the current the controls and calibrator acceptable ranges.
  - iv. The acceptable calibrator values used should be within 2 SD of the assigned value.
- b. Pooled High Calibrator
- i. Single level high calibrator was prepared from pooled EDTA whole blood purchased from Aalto Scientific. Refer to Aalto Scientific product insert for additional details.
  - ii. The blood was dispensed into 250  $\mu\text{L}$  aliquots under refrigerated conditions. Batches of these high calibrators (aliquots) were assigned a lot number, date the high calibrators was made (and received from Aalto) and stored at  $-70^{\circ}\text{C}$  on the same day. The remaining aliquots were assigned the same lot number/date and were placed in a cryogenic (liquid nitrogen) tank at  $-196^{\circ}\text{C}$  in freezer boxes in the same day.<sup>5-7</sup>
  - iii. High calibrator HbA1c values were assigned by twenty interassay determinations along with the previous lot of calibrator. Refer to QC and Calibrator's binder and the controls and calibrator color key table (locate at the bench) for the current the controls and calibrator acceptable ranges.
  - iv. The acceptable calibrator values used should be within 2 SD of the assigned value.
- c. Preparation and Stability – Aliquots from Liquid Nitrogen to  $-70^{\circ}\text{C}$ .
- i. Removed one box of calibrators from the liquid nitrogen phase to the vapor nitrogen phase for at least 2 hours. After 2 hours, transfer box into the  $-70^{\circ}\text{C}$  freezer for at least 2 hours to allow materials to acclimate. If box is already in the vapor nitrogen phase, move box into the  $-70^{\circ}\text{C}$  freezer and allow materials to acclimate.
  - ii. After the acclimation period, transfer 50 tubes (while working in the freezer) into a box labeled the material's name, date removed from liquid nitrogen, and with an expiration date 365 days (one year) into the future.
  - iii. Return the box with the remaining calibrator aliquots to the vapor nitrogen phase of the liquid nitrogen.
  - iv. Low and high calibrator aliquots are pulled from freezer daily and placed in the refrigerator ( $2-8^{\circ}\text{C}$ ) at start of the day. Prior to use, thawed materials at room temperature, and mixed by inversion gently.
  - v. WB calibrations are diluted with hemolysis & wash solution to obtain a Total Area (TA) in the range of 500-

4000. Calibrators are discarded after daily use.
2. Calibration 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 a new reagent lot.
    - f. After a filter change.
    - g. After analyzer maintenance.
    - h. Refer to Operator's Manual for additional troubleshooting advice.<sup>1</sup>
  3. Calibration Procedure:
    - a. Verify that there is sufficient volume of Elution Buffers, Hemolysis & Wash Solution and at least 400  $\mu$ L 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 low calibrator in position 1 (on the left) and high calibrator next to it in position 2.
      - i. Press the START key to begin the calibration.
      - ii. 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.
    - f. Current calibrator value assignments can be found in the Quality Control Binder.
  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.
    - a. Verify the acceptability of the chromatogram. Refer to Operator's Manual for additional details. If the calibration is unsuccessful, recalibration is required. Patient aliquots are not assayed until the calibration results are acceptable.
    - b. A Calibration Error message appears and the run aborts if;
      - i. The two SA1c% results for Calibrator 1 differ by 0.3% or more.
      - ii. The two SA1c% results for Calibrator 2 differ by 0.3% or more.
      - iii. Any of the four calibrator results differ from its assigned value by  $\pm$  30% or more.

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

1. 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. Replace buffer if necessary. All changes in lot numbers of buffers are to be recorded on the Tosoh Diary Sheet, and in the Tosoh G8 daily lot info. spreadsheet.
  - 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.
2. 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:
    - i. Wipe outside of tip with gauze wetted with distilled water.

- ii. Insert tip of autodilutor into blood specimen and press the button on top of the handle to draw 10ul of specimen into the tip.
    - iii. Insert tip into corresponding 1.5mL sample cup and press button again to dispense sample and reagent into sample cup.
    - iv. Wipe outside of tip again with gauze wetted with distilled water.
    - v. 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 PLC in position 1 (on the left) and PHC in position 2.
  - c. Load controls: Controls are performed at the beginning of the run, every 50 samples, and at the end of a run.
  - d. Place controls in the rack with low in position 3 and high in position 4.
  - e. Place low and high controls in the end positions.
  - f. Load samples.
    - i. Mix each sample by gently inverting each capped sample tube.
    - ii. 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.
    - iii. Position each rack in the rack guide, starting with the right side.
    - iv. Place a blank rack after the last rack of samples. The blank rack serves as an end marker.
3. 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".
  - 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.<sup>1</sup>
    - iv. The acceptable retention time for A0 is 0.87 – 0.91.<sup>1</sup>
    - 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.<sup>1</sup>
  - Samples with TA > 4000; reassay the specimen using more Hemolysis & Wash Solution.<sup>1</sup>
- vi. Repeat any specimens with %HbA1c values less than 4.0% or greater than 14.0% for verification.
  - 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”. Enter into the Primus data base the specimen ID, and in the comment section “X-PK”. Also, record the specimen into the Primus Sample Tracking from Tosoh G8 log and rerun by the Trinity Premier boronate affinity method. Report the boronate affinity HbA1c result.
  - 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. Enter into the Primus database the specimen ID, and in the comment section “Hemoglobin D”. Also, record the specimen into the Primus Sample Tracking from Tosoh G8 log and rerun by Trinity Premier boronate affinity method. Report the boronate affinity HbA1c result.
  - 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. Enter into the Primus database the specimen ID, and in the comment section “Hemoglobin S”. Also, record the specimen into the Primus Sample Tracking from Tosoh G8 log and rerun by Trinity Premier boronate affinity method. Report the boronate affinity HbA1c result.
  - 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. Enter into the Primus data base the specimen ID, and in the comment section “Hemoglobin C”. Also, record the specimen into the Primus Sample Tracking from Tosoh G8 log and rerun by Trinity Premier boronate affinity method. Report the boronate affinity HbA1c result.
  - xi. Samples with heterozygous HbE will exhibit an additional small peak between the SA1C and A0 peaks. The instrument will designate the HbE peak as PHV-E. The chromatogram is labeled with “HbAE”, and the %HbA1c cannot be reported for these specimens. Enter into the Primus data base the specimen ID, and

in the comment section “Hemoglobin E”. Also, record the specimen into the Primus Sample Tracking from Tosoh G8 log and rerun by Trinity Premier boronate affinity method. Report the boronate affinity HbA1c result.

- xii. Samples with chromatograms indicating that there is a presumptive variant peak that is not flagged as HbS, HbC, HbE, and HbD should be marked as “ABN.” Enter this sample ID (with the ABN indication) into the Primus database and record the specimen into the Primus Sample Tracking from Tosoh G8 log and rerun by Trinity Premier boronate affinity method. Refer to the supervisor or delegate for if you have questions interpreting chromatograms.
- xiii. Samples with chromatograms showing the presence of both a variant hemoglobin and HbF > 10 % should not be reported.
- xiv. G8 results  $\leq 10$  % HbA1C that have a LA1C  $\geq 1/2$  SA1C are reflexed to the boronate affinity HPLC method for verification due to potential variants. Enter into the Primus data base the specimen ID, G8 result, and in the comment section “the LA1c and A1c result”. Also, record the specimen into the Primus Sample Tracking from Tosoh G8 log and rerun by Trinity Premier boronate affinity method.
- xv. Chromatograms that are not flagged as abnormal, but appear questionable under the supervisor (or delegate) discretion should be labeled “primus checks”. Enter into the Primus data base the specimen ID, G8 result, and in the comment section “Primus Check”. Also, record the specimen into the Primus Sample Tracking from Tosoh G8 log and rerun by the rerun by Trinity Premier boronate affinity method.
- xvi. Fetal hemoglobin (HbF) up to 38.4% 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. <sup>b, d</sup> 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:  $\% \text{ SA1c} = (((\text{SA1c Area} / (\text{A1a Area} + \text{A1b Area} + \text{SA1c Area} + \text{A0 Area})) \times 100) \times \text{Slope} + \text{Y Intercept})$ .
- xvii. Any abnormal peak pattern consistent with a homozygous variant hemoglobin (e.g. HBSS) or double heterozygous variant (e.g. HbSC) should be not be reported. This result is considered unreportable. The report is noted with the comment, “An abnormal peak pattern consistent with a homozygous or double heterozygous variant hemoglobin was detected. This condition is known to affect the average lifespan of red blood cells, so the HbA1c test should not be used for this patient. An alternative test to measure glycemia is fructosamine/glycated serum protein.”

- xviii. 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.
4. Reporting Results: 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." Chromatography 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. 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.
    - e. In the NHANES database, select "Enter Results:", then "A1c Results-Pull" to display the NHANES samples to be imported from the G8.
    - f. Select the results to be imported then select "Commit Results", then "Close".
    - g. Select "Reports", then "Print Check Sheet" and enter the batch number to print the check sheet.
    - h. Submit check sheet(s), chromatography, and result list printouts along with completed diary sheet to supervisor for verification.
    - i. Supervisor will verify acceptability of entire run based upon controls, and acceptability of individual results based upon chromatography.
    - j. When satisfied, the supervisor will clear the results in the NHANES database.
  5. 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.
  6. 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. Linearity is performed every 6 months. The established AMR is 3.1 – 19.5%.

7. Supervisor Responsibility:
  - a. The supervisor or delegate ensures quality control passes within the acceptable ranges prior to releasing patient results.
  - b. The supervisor or delegate checks every individual chromatogram to ensure all peaks are resolved and reportable.
  - c. All chromatograms requiring further evaluation are noted and are not uploaded into the database.
8. Procedure Notes
  - a. To avoid an error condition during calibration, be sure to place PLC and PHC in the first sample rack in positions 1 and 2 respectively.
  - b. 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.
  - c. The reagents must be at room temperature prior to use.
  - d. 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.
  - e. The relationship between HbA1c results from NGSP network and the IFCC network is expressed by using the following equation:  $NGSP (\%) = 0.09148 \times IFCC (mmol/mol) + 2.152^2$
  - f. 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.
  - g. 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

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. Linearity is performed every 6 months. The established AMR is 3.1 – 19.5%.

## 10. QUALITY CONTROL (QC) PROCEDURES

## 1. Quality Control

a. Quality Control Preparation: Donors are recruited and compensated for their donation of blood. Blood products are pooled together, mixed for at least 30 min, and aliquoted under refrigerated conditions, see below.

### i. Pooled Low Control

- 1) Single level low control was prepared from K<sub>2</sub>EDTA whole blood whole blood were drawn from known non-diabetic individuals (Normal level HbA1c).
- 2) The blood specimen were pooled, dispensed in 50 µL aliquots into 400µL microtubes under refrigerated conditions. Batches of low controls (aliquots) were assigned a lot number, preparation date (date the controls were collected), and stored at -70°C on the same day (working stock for daily usage, no more than 50 tubes). The remaining aliquots were assigned the same lot number/date and placed in a cryogenic (liquid nitrogen) tank at -196°C in freezer boxes in the same day (long term storage).

### ii. Pooled High Control

- 1) The elevated (Abnormal) HbA1c level pooled whole blood (K<sub>2</sub>EDTA) controls were purchased from Aalto Scientific. Refer to Aalto Scientific product insert for additional details.
- 2) The blood was dispensed into 250 µL aliquots under refrigerated conditions. Batches of high control, were assigned a lot number, dated with the day the controls were collected (prepared), and aliquots were stored at - 70°C on the same day (working stock for daily usage, no more than 50 tubes). The remaining high control aliquots were assigned the same lot number/date and were placed in a cryogenic (liquid nitrogen) tank at -196°C in freezer boxes on the same day (long term storage).

b. Preparation and stability.

- i. Removed one box of calibrators from the liquid nitrogen phase to the vapor nitrogen phase for at least 2 hours. After 2 hours, transfer box into the -70°C freezer for at least 2 hours to allow materials to acclimate. If box is already in the vapor nitrogen phase, move box into the -70°C freezer and allow materials to acclimate.
- ii. After the acclimation period, transfer 50 tubes (while working in the freezer) into a box labeled the material's name, date removed from liquid nitrogen, and with an expiration date 365 days (one year) into the future.
- iii. Return the box with the remaining calibrator aliquots to the vapor nitrogen phase of the liquid nitrogen.
- iv. Low and high control aliquots are pulled from freezer daily and placed in the refrigerator (2-8 °C) at start of the day. Prior to use, thawed materials at room temperature, and mixed by inversion gently.

- v. WB controls are diluted with hemolysis & wash solution to obtain a Total Area (TA) in the range of 500-4000. Controls are discarded after daily use.
- c. Mean and Ranges
  - i. Daily means and ranges are 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.
  - iv. Current Control value assignments and limits can be found in the Quality Control Binder located in room M767.
- d. Tolerance Limits
  - i. Analytical Batch Quality Control – Daily
    - 1) 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 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. Results are not released until the quality control specification are acceptable.
  - ii. Levey-Jennings Plots – Monthly
    - 1) Mean chart—Plots the mean values for each control in the 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) The Laboratory Director or delegee reviews these on a monthly basis.
- e. Routine Quality Control Testing
    - i. Normal and Abnormal level (elevated) controls are run at the beginning of a run, every 50 samples, and at the end of the run.
    - ii. Sample QC—Two percent of HbA1C specimens are randomly selected and reanalyzed. Duplicate results are hand written on the laboratory results worklist and reviewed for accuracy by trained personnel. If the difference in %HbA1c between the duplicate is greater than 4% (relative) or 0.2% HbA1C (whichever is greater) of the original HbA1c value, the specimen is reanalyzed and the chromatograms and instruments from both the original and duplicate runs are investigated.
  - f. Inter-instrument QC
    - i. 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 the estimate of the standard deviation of the difference in sample replicates that must not exceed 0.229 (99th percentile of the sampling distribution around a target SD of 0.15).<sup>2</sup>
    - ii. Comparison between Tosoh G8 and Trinity *ultra*<sup>2</sup>/Premier instruments:
      - 1) GHBQC procedure is followed for the comparison of results (n = 30 – 50 per month) between the Tosoh G8 HPLC HbA1c method and its backup method, Trinity *ultra*<sup>2</sup>/Premier HPLC HbA1c. Criteria for Pass/Fail: Bland/Altman: +/-0.70, Y at X: +/-0.30, Syx: 0.35
      - 2) Results are reviewed monthly by the Lab Director.
  - g. Proficiency Testing
    - i. College of American Pathologists (CAP) survey:
      - 1) Three times a year Diagnostic Diabetes Lab (DDL) will receive Proficiency Testing (PT) specimens from CAP. The testing personnel who receives these samples should store and handle these specimens according to the instructions provided. CAP PT are tested as patient samples (and named according to their sample IDs) within the routine lab workload, results are recorded on the forms included, and then submitted online by the lab supervisor or delegate. Interlaboratory communication about proficiency testing samples or results is NOT allowed until after the close of a PT event (after the submission deadline has passed).
      - 2) Technologists should rotate testing of proficiency survey samples (during different PT events), so that the same tech is not running all the PT surveys. Refer to DDL's Quality Management Program for additional details relating to PT survey handling and reporting.

- 3) Due to a recent CMS directive, our institution is only allowed to order and result one PT survey per analyte; laboratories are not permitted to test PT samples on multiple instruments. During a PT event the primary instrument should be utilized (if this is how testing is routinely performed). Proficiency Surveys should be rotated among primary instruments during different PT events.
  - 4) Once the CAP PT results are submitted by the lab supervisor or delegee, the proficiency samples are placed and maintained under frozen conditions (-70 °C) until after the close of that PT event. The PT samples should then be retested on the other G8 HPLC platforms (that were not used during the PT event) and the boronate affinity methods (Premier) after the submission deadline.
  - 5) Results from all DDL HbA1c methods are compared to the assigned target values obtained from the PT evaluation report (COM.04250 Comparability of Instruments/Methods) using the HbA1c CAP Total Allowable Error (pass/fail criteria) of  $\pm 6\%$ . Multiple instrument Comparison PT results from each survey are entered in the "HPLC Multiple Instrument Comparison" spreadsheet found on the H drive (H:\DDL\CAP\Intra-instrument Comparison).
  - 6) The Lab director, manager or delegate will review the results from this study and approve if the evaluation is satisfactory. Any discrepant results observed outside of the acceptable criteria are investigated further.
- h. Carryover Studies- Performed following manufacturer's experimental design and guidelines;<sup>14-15</sup>
- i. Prepare two specimens, one with a very high %HbA1c and one with a very low %HbA1c value.
  - ii. Aliquot these specimen: 11 with low %HbA1c concentration and 10 with high %HbA1c concentration.
  - iii. No other specimens or test should be assayed on the instrument and the samples should be assayed using the below carryover sample order: 3 L (low) / 2 H (high)/ 1 L /2 H/4 L/2 H /1 L/2 H/1 L/2 H/1 L
  - iv. After analyzing the low and high specimens in the above order, enter the results in EP Evaluator using the carryover program. Refer to Teitz (ed). *Textbook of Clinical Chemistry* , page 421. WB Saunders Co, Philadelphia, 1986 and the EP Evaluator carryover report interpretation guide for additional details.<sup>14-15</sup>
  - v. The carryover test passes if the results of the High-low sequences are statistically identical to the results of the low-low sequences (three times the SD of the low-low result – the SD that would be expected if no high results were measured). The results of the low-level sample should not be affected by the high-level sample.

Carryover studies should be performed roughly every year or after major instrument maintenance.

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

- i. If control results are outside of the acceptable limits, sample results should not be reported. Samples should be return to optimal storage conditions until all QC issues are resolved. QC Corrective actions could include repeating of QC (of the same lot), performing a recalibration procedure, changing out the appropriate reagents, retesting patient samples from a previously acceptable run (to check instrument performance), and performing analytical precision studies (to test the integrity of the control material in use, etc.).
- ii. QC results must be acceptable prior to releasing patient results. If QC results are still unacceptable after performing corrective actions, call the appropriate technical vendor hotline for additional troubleshooting advice and alert the supervisor or delegate immediately.
- iii. All Corrective Action/Preventive Action (CAPA) events concerning out of range controls or calibrators, or rejected runs that occur at the time of testing, should be documented (noted on the daily diary sheet for that particular assay and QC Corrective Action form) and immediately communicated (verbally and by email) to the Lab Director or Manager .
- iv. If the calibration is unsuccessful, recalibration is required. Patient aliquots are not assayed until the calibration results are acceptable.
- v. QC and calibration results must be considered acceptable prior to reporting patient samples.

## **12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS**

1. Dilution studies demonstrate that the assay is linear from a Total Area of 500-4000.
2. 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.).
3. 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 HbA1c levels.<sup>1</sup>
4. The life span of red blood cells is lengthened in polycythemia or postsplenectomy patients. Specimens from such patients may exhibit increased HbA1c levels.<sup>1</sup>
5. Interference:

- a. The presence of hemoglobin variants (e.g. HbC, HbF >38.4%, HbE, HbD, HbS, etc.) may interfere with HbA1c results. <sup>17</sup>
- b. Refer to Operator's manual Interference section for additional details.

### 13. REFERENCE RANGES (NORMAL VALUES)

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.

**Interpretation:** The level of HbA1c is an index of average blood/plasma glucose during the preceding 3-4 months and has strong predictive value for diabetes complications. HbA1c  $\geq$  6.5% is diagnostic for diabetes. HbA1c 5.7-6.4% indicates increased risk for diabetes Note: Any condition that shortens erythrocyte survival or decreases means erythrocyte age (e.g. hemolytic anemia) will falsely lower HbA1c results regardless of the assay method (ADA Clinical Practice Recommendations).

### 14. CRITICAL CALL RESULTS ("PANIC VALUES")

N/A

### 15. SPECIMEN STORAGE AND HANDLING DURING TESTING

Any specimens not analyzed on the day of arrival in the laboratory are stored in the refrigerator (2°C - 8°C). Upon completion of analysis, 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 3 G8 instruments for performing glycohemoglobin (HbA1c). If none are available for use, the specimens are stored refrigerated at 2 -8°C until testing can be performed.

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

NHANES data files with results are exported from the NHANES database in the specified format and uploaded to Westat via secure transfer weekly.

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

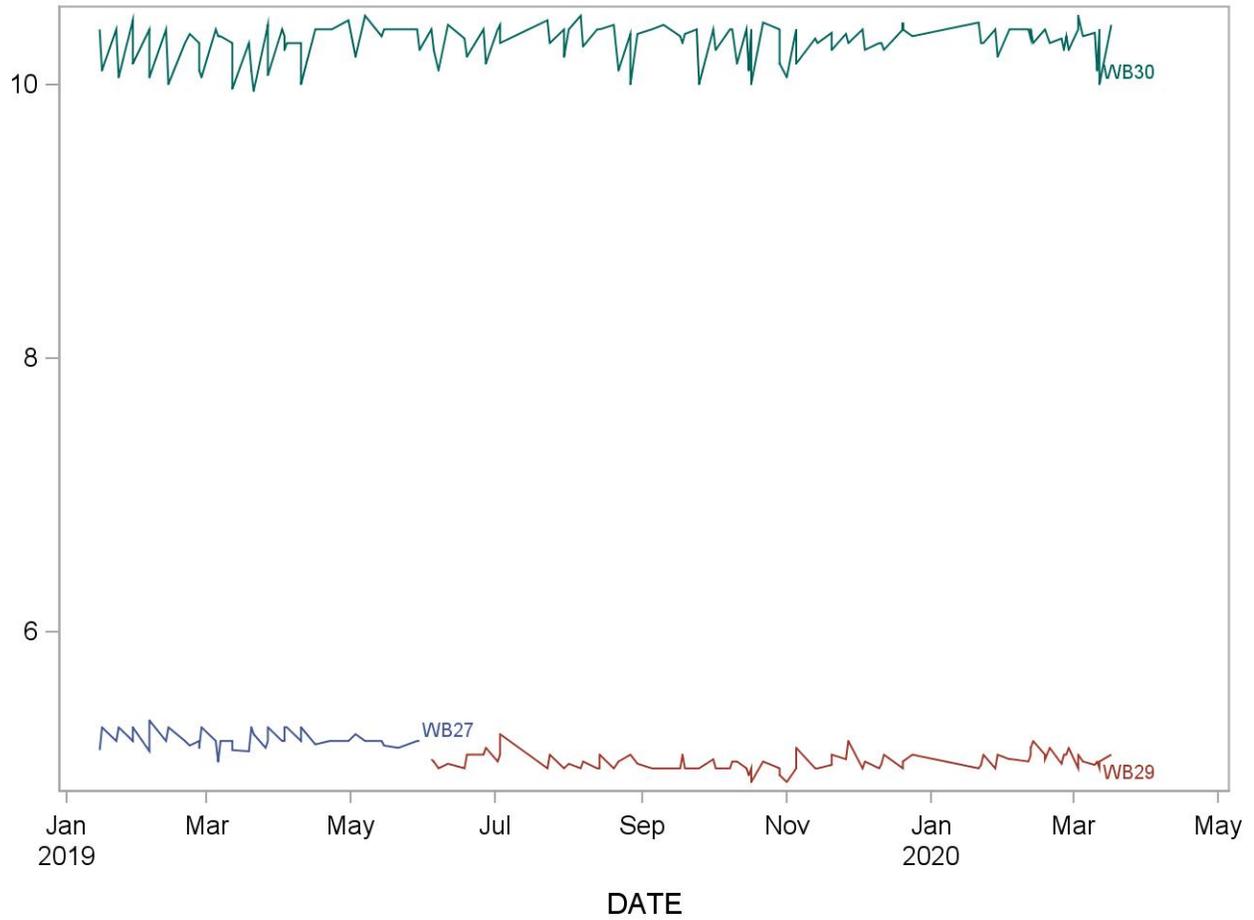
All shipments are recorded on the NHANES Shipping Log upon receipt. Actions taken during the course of analysis, result reporting, and specimen retention are also recorded on the log. Specimens are stored frozen at -70°C or colder after analysis; specimen locations are recorded according to sequential DDL accession number and box number. After one year specimens may be discarded.

**19. SUMMARY STATISTICS AND QC GRAPHS**

See following pages.

### 2019-2020 Summary Statistics and QC Chart LBXGH (Glycohemoglobin (%))

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
WB27	60	15JAN19	30MAY19	5.23	0.07	1.4
WB30	189	15JAN19	17MAR20	10.27	0.14	1.4
WB29	129	04JUN19	17MAR20	5.06	0.07	1.3



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