Laboratory Procedure Manual

Analyte: **Glycohemoglobin (HbA1c)**

Matrix: **Whole Blood**

Method: **Bio-Rad D-100 High Performance Liquid Chromatography (HPLC) Glycohemoglobin Analyzer**

Revised Date: **Dec 5th, 2023**

As performed by: **Diabetes Diagnostic Laboratory**
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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_L	LBXGH	Glycohemoglobin (%)

1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

a. Test Principle: The D-100 test utilizes principles of ion-exchange high-performance liquid chromatography (HPLC). The samples are automatically diluted on the D-100 and injected into the analytical cartridge. The D-100 delivers a programmed buffer gradient of increasing ionic strength to the cartridge, where the hemoglobins are separated based on their ionic interactions with the cartridge material. The separated hemoglobins then pass through the flow cell, where changes in the absorbance at 415 nm are measured. The D-100 software collects raw data from each analysis and calculates HbA1c values based on a bi-level calibration curve. The HbA1c area is calculated using an exponentially modified Gaussian (EMG) algorithm. A sample report and a chromatogram are generated for each sample.

b. Clinical Significance: Diabetes mellitus is a condition characterized by hyperglycemia resulting from the body's inability to use blood glucose for energy. In Type 1 diabetes, the pancreas no longer makes insulin and therefore, blood glucose cannot enter the cells to be used for energy. In Type 2 diabetes, either the pancreas does not make enough insulin, or the body is unable to use insulin correctly. The direct and indirect effects of hyperglycemia on the human vascular system are the major source of morbidity and mortality in both Type 1 and Type 2 diabetes. These effects include macrovascular complications (coronary artery disease, peripheral arterial disease, and stroke) and microvascular complications (diabetic nephropathy, neuropathy, and retinopathy). Diabetes mellitus affects >8% of the world population. HbA1c testing has been recommended for the diagnosis of Type 2 diabetes by the International Expert Committee (IEC), the American Diabetes Association (ADA), and the World Health Organization (WHO), which recommend a diagnostic threshold of ≥6.5% HbA_{1c}. HbA1c testing has also been recommended for the identification of individuals at increased risk for developing diabetes (pre-diabetic). The ADA has defined the HbA1c range for pre-diabetes as 5.7–6.4%.[4] Detection and treatment of pre-diabetes may reduce or eliminate the risk of developing Type 2 diabetes and related complications.

Therapy for diabetes requires the long-term maintenance of a blood glucose level as close as possible to a normal level, minimizing the risk of long-term vascular consequences. A single fasting blood glucose measurement is an indication of the patient's immediate past condition (hours) but may not represent the true status of blood glucose regulation. The measurement of hemoglobin A_{1c} (HbA_{1c}) every two to three months has been accepted as a measure of glycemic control in the care and treatment of patients with diabetes mellitus.

HbA_{1c}, the glycohemoglobin of interest, is formed in two steps by the nonenzymatic glycation of HbA. The first step is the formation of an unstable aldimine (labile A_{1c}, or pre-A_{1c}), a reversible reaction between the carbonyl group of glucose and the N terminal valine of the β-chain of hemoglobin. Labile A_{1c} formation is directly proportional to the blood glucose concentration. During red blood cell circulation, some of the labile A_{1c} is converted (Amadori rearrangement) to form a stable ketoamine, HbA_{1c}.

The D-100 HbA1C test is based on chromatographic separation of HbA_{1c} on a cation exchange cartridge. Separation is optimized to minimize interferences from hemoglobin variants, labile A_{1c}, and carbamylated hemoglobin. Please refer to Limitations of the Procedure for more information. The D-100 HbA_{1c} test also offers automatic sampling from a primary whole blood tube, followed by sample dilution, and an analysis time of 45 seconds per sample.

2. SAFETY PRECAUTIONS

a. Universal Safety Precautions should be practiced, while working in the lab. Proper PPE is enforced, which includes wearing gloves, face masks/shields, lab coats, protective eye wear such as goggles, and closed toe shoes are required when 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.
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 Oxivir 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 Oxivir disinfectant or 10% bleach. Refer to the Lab Safety Manual located in room M764 (for additional details).

   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 specimen 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 such as eye goggles, and protective clothing.

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

3. COMPUTERIZATION; DATA SYSTEM MANAGEMENT

Data are maintained on a secured Microsoft Access / Microsoft SQL server client-server system via 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

a. Patient Preparation
   i. No special conditions, such as fasting or special diets, are required for this test.

b. Specimen Type and Stability
   i. Collect whole blood specimen in vacuum collection tube containing EDTA anticoagulant (K2/K3-EDTA).
   ii. Specimen Storage:
      1. Room Temperature (15-35°C): 1 Day
      2. Refrigerated (2-8°C): 7 Days
      3. Freezer (-20°C), (-70°C): 7 Days, 6 Months

      Note: Prediluted samples are stable for 3 hr. at 15-35°C.
   iii. The minimum volume required for analysis directly from the collection tubes is 1 mL of whole blood. If the height of the sample (blood) in the tube appears to be ≤ 1 cm (≤ 500 µL), the sample may need to be prediluted 1:300 prior to analysis.
   iv. Refer to the sample preparation section below for handling specimens that require off-line dilutions.

c. Unacceptable specimen (for testing) criteria:
   i. Clotted specimens should not be placed directly on the analyzer, but they may be diluted offline if care is taken to avoid adding any of the clot into the dilution.
   ii. Specimen type and stability not listed above.
   iii. Samples not suitable for testing should be rejected. Refer to the Laboratory - Specimen Rejection - Guideline for additional details.

iv. In case of sample rejection, immediately inform lab supervisor or approved delegate so that NHANES can be notified.

d. Handling Conditions:
   i. Samples are to be kept at 2-8°C until analyzed. Analysis should occur within 24 hr. of receipt.
   ii. Once received and prepared for analysis, specimens are to be returned to 2-8°C storage. Once testing is completed and results are considered acceptable, samples should immediately be stored under -70°C conditions.

5. PROCEDURES FOR MICROSCOPIC EXAMINATION

Not applicable for this procedure.

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

a. Equipment
   i. Bio-Rad D-100 Analyzer and System.
   ii. Hamilton Auto dilutor with 2.5 mL and 25 µL syringes.

b. Equipment Maintenance: Refer to the D-100 manual for Start-up, Operation, Shut Down, and Maintenance procedure for additional details.
   i. Installing a New Analytical Cartridge
      1. Ensure the instrument is in Sleeping state (Utilities/Manual Operations/General/Sleep).
      2. Open the cartridge holder door.
      3. Remove the old cartridge.
      4. Insert the new cartridge.
      5. Close the cartridge holder door. Test parameters are automatically updated.
   ii. Installing a New Prefilter
      1. Ensure the instrument is in Sleeping state (Utilities/Manual Operations/General/Sleep).
      2. Open the prefilter holder door.
      3. Remove the old prefilter.
      4. Insert the new prefilter.
      5. Close the prefilter holder door. The prefilter information is automatically updated.
   iii. Replacing an Empty Reagent Bottle
      1. Open the reagent compartment door.
      2. Insert a new bottle of the same reagent, pushing it into the compartment until it latches in place.
      3. Press the screen on the bottle being removed, then press the remove button. After the system has depressurized, move on to the next step.
      4. Remove the empty bottle by pushing it in, lifting it up over the ledge, and pulling it out by the handle.

a. NOTE: The reagent information is automatically updated when the RFID is read. The instrument will 'signal yellow' when a reagent change is necessary. Install the new bottle in the open spot. The reagent information is automatically updated. The instrument will automatically switch to the new buffer when the old buffer level changes from yellow to red.

5. Close the reagent compartment door.

iv. Pipette Preventative Maintenance

1. Hamilton Autodilutor 500/600
    a. Instrument should be cleaned and inspected for proper functioning daily.
    b. The Autodilutor should be calibrated at least annually.

v. Adjustable Volumetric Pipettes

1. After each use, the pipette should be wiped with disinfectant with soaked gauze.
2. Pipettes are calibrated annually by a trained field service personnel.

c. Materials and Stability:

i. Reagents – Supplied by Bio-Rad Laboratories, Inc. (4000 Alfred Nobel Drive Hercules, CA).

1. Analytical Cartridge
    a. Stable until expiration date when stored at 2-8°C.
    b. The cartridge can be used immediately after removing from refrigerator.
    c. When installed on the instrument, cartridge is stable for 90 days on the instrument at 15 - 35°C.
    d. Replace at 90 days or 10,000 tests/injections.
2. Prefilter
    a. Stable until expiration date when stored at 2-8°C.
    b. The Prefilter can be used immediately after removing from the refrigerator.
    c. When installed, the prefilter is stable for 90 days on instrument at 15-35°C.
    d. Replace the prefilter at 90 days or 2000 tests.
3. Elution Buffers A, Elution Buffer B, and Wash solution
    a. Stable until expiration dates when stored unopened at 15-35°C.
    b. After installing the bottles on the instrument, reagents are stable for 90 days on instrument at 15-35°C.
    c. The Elution Buffers are interchangeable within cartridge resin lots. All lots of Wash solution are interchangeable.
4. Sample Diluent
    a. Stable until expiration date when stored unopened at 15-35°C.
    b. After opening, stable for 90 days after opening at 15-35°C.
5. Calibrator Pack
    a. Stable until expiration date when stored unopened at 2-8°C.

b. Once reconstituted by the system, the pack is stable for 24 hours after initial use when stored at 2-8°C. The pack may be used for a second calibration within this period.

6. Cleaning Tube
    a. Stable until expiration date when stored unopened at 15-35°C.
    b. Refer to SDS for information on hazards and precautions with this material.
    c. Note:
        i. This HPLC method does not use extracted standards.
        ii. If Elution Buffers, Wash Solution, or Sample Diluent were frozen during shipment, allow them to reach to room temperature (15-35°C) and mix each bottle by gently inverting before use.
        iii. Do not use any reagents that show signs of external leakage.
7. Reagent Labeling – reagents (e.g., buffers, wash), calibrators, controls, and solutions should be traceably identified to indicate the following:
    a. Content and expiration date.
    b. Storage requirements.
    c. The below should be followed for working reagents (open reagents in use for testing):
        i. Controls and calibrators obtained from the freezer for daily use should be initialed and dated with the pulled date.
        ii. Prior to processing controls and calibrators, sample cups should be traceably labeled (with the name of the control or calibrator), dated, and initialed (tech responsible for adding hemolysis wash to the sample cups). Refer to the controls and calibrator color key table (locate above the BSC in room M764).
        iii. Prediluted matrix appropriate whole blood controls and calibrators are stable for 3 hr. at 15-35°C. Controls and calibrators should be made fresh in the AM and the PM, prior to each analytical run. These aliquots should be discarded after the AM and PM shifts.
        iv. Sample diluent bottles should be labeled with an open date, expiration date (from open or preparation date), and tech's initials.
        v. On-board/working reagents should be traceably identified to indicate content and expiration date.

d. Calibration
    i. Calibrator Preparation: EDTA whole blood tubes are pooled together, mixed for at least 30 min, and aliquoted under refrigerated conditions, see below.
        1. Low Calibrator
            a. Single level low calibrator was prepared from pooled EDTA whole blood specimens with normal HbA1C levels (Reference Range).
            b. 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, labeled with the preparation date, and stored at -70°C on the same day (working stock for daily usage, not more than 30 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 the same day (long term storage).
            c. Low HbA1c calibrator values were established by performing at least twenty runs in duplicate measurements, along with the previous calibrator lot, to establish the target tolerance limits.
            d. The mean of these analyses was used as the assigned value (or rounded to the nearest tenth decimal place if the instrument allows only single decimal place precision for calibrator assigned values).
            e. The acceptable calibrator values used should be within ±3 standard deviations (SD) of the assigned value.
            f. Calibrators are high-quality materials that are traceable to the NGSP (http://www.ngsp.org/) and IFCC networks.
        2. High Calibrator
            a. Single level high calibrator was prepared from pooled EDTA whole blood purchased from Aalto Scientific. Refer to Aalto Scientific product insert for additional details.
            b. The blood was dispensed into 250 µL aliquots under refrigerated conditions. Batches of these high calibrators (aliquots) were assigned a lot number, date the high calibrators were made (and received from Aalto) and stored at -70°C on the same day (working stock for daily usage, no more than 30 tubes). The remaining 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).

c. High HbA1c calibrator values were established by performing at least twenty runs in duplicate measurements, along with the previous calibrator lot, to establish the target tolerance limits.
d. The mean of these analyses was used as the assigned value or rounded to the nearest tenth decimal place if the instrument allows only single decimal place precision for calibrator assigned values.
e. The calibrator values used should be within ±3 standard deviations (SD) of the assigned value.
f. Calibrators are high-quality materials that are traceable to the NGSP (http://www.ngsp.org/) and IFCC networks.

3. Preparation and Stability - Aliquots from Liquid Nitrogen to -70°C.
a. Move one box of calibrators from the liquid nitrogen liquid phase to the vapor phase of the liquid nitrogen 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 the box is already in the vapor phase of liquid nitrogen, move box into the -70°C and allow materials to acclimate.
b. After the acclimation period, transfer 30 tubes (while working in the freezer) into a box labeled with the material's name, date removed from nitrogen, and an expiration date 7 months into the future.
c. Return the box with the remaining calibrator aliquots to the vapor phase of the liquid nitrogen.
d. Low and High calibrators are pulled from the freezer as required and placed in the refrigerator (2-8°C) at the start of the day. Prior to use, thaw materials at room temperature and mix gently by inversion.
e. Calibrators should be discarded after use.

e. Quality Control
i. Quality Control Preparation: Donors are recruited and compensated for their blood. Blood products are pooled together, mixed for at least 30 min, and aliquoted under refrigerated conditions, see below.
1. Pooled Low Control:
a. Single level low control was prepared from EDTA whole blood drawn from non-diabetic individuals (normal level HbA1c). Refer to the QC and Calibrations' Manual for the current QC lot in use.

b. The blood specimens were pooled, dispensed in 50 µL aliquots into 400 µL microtubes under refrigerated conditions. Batches of low controls (aliquots) were assigned a lot number, preparation date (collection date), 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 on the same day (long term storage).
c. Controls are high-quality materials that are traceable to NGSP (www.NGSP.org) and the IFCC networks.

2. Pooled High Control
   a. The elevated (abnormal) HbA1c level pooled whole blood (EDTA) control was purchased from Aalto Scientific.
   b. The blood was dispensed into 100 µL aliquots. Batches of the high control were assigned a lot number, preparation date, and stored at -70°C on the same day. 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 the freezer boxes on the same day (long term storage).
   c. Controls are high-quality materials that are traceable to NGSP (www.NGSP.org) and the IFCC networks.

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

7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

a. Calibration Procedure:
   i. For the initial calibration of a new analytical cartridge, follow manufacturer's instructions in the Operator's Manual or Quick Guide for instructions on running the Bio-Rad Calibrator Pack.
   ii. After initial calibration with Bio-Rad Calibrator Pack, calibrate with in-house calibrators.
      1. Predilute each in-house calibrator 1:300 using the Hamilton Autodilutor to dilute 5 µL of whole blood with 1.5 ml sample diluent.
      2. The D-100 instrument must be in Sleeping or Standby state
      3. Go to the Calibration screen and touch 'Calibrate Now'
      4. In the Calibration dialog box, touch Load Stat Area. The stat rack is moved to the loading position.
      5. Load the stat rack by placing the blank vial in the first spot, the low calibrator in the second spot, and the high calibrator in the third spot, using a microvial adapter for each position.
      6. Touch Load Stat Area. The stat rack is then loaded into the Stat Area.
      7. Enter the required calibrator information (i.e., name, assigned values) and select Calibrate without reconstituting checkbox.
      8. Touch Calibrate Now.
      9. After the Stat Area samples are finished being processed, touch Open.
      10. Remove the samples from the stat rack.

b. Frequency
   i. Calibration/Recalibration is to be performed: When the column is initially installed, system should be calibrated using manufacturer's analytical cartridge/calibration pack. Calibration using Bio-Rad analytical cartridge should be performed at minimum every 90 days or 10,000 injections, and when one of the following occurs:
      1. Control values fall outside of the acceptable range.
      2. QC is observed to drift.
      3. After major maintenance of the instrument.
      4. Refer to the operator's manual for additional troubleshooting advice.
      5. The D-100 should be calibrated using NGSP calibrators:
         a. Weekly
         b. Immediately after the system is calibrated with Bio-Rad analytical cartridge/calibration pack.

ii. Calibration Acceptability Criteria
1. When the calibration procedure is complete, the instrument automatically accepts or rejects the calibration results
2. Controls should be analyzed immediately after calibration.
3. If either the instrument rejects the calibration or the controls fall outside acceptable criteria, the instrument should undergo troubleshooting to determine the potential causes prior to recalibration.
4. Calibration records are maintained on the instrument.

c. Calibration Verification
Calibration Verification materials are purchased through CAP LN15 Hemoglobin HbA1c Calibration Verification survey. Testing is performed every six months and results are considered acceptable within a Total Allowable Error of 12%

8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

a. Procedure - Stepwise:
i. Instrument setup for the Bio-Rad D-100
1. Visually verify that sufficient levels of reagents are loaded onto the machine. If any reagent's status is displayed in yellow, a new bottle of that reagent should be installed in the appropriate slot on the instrument. All changes in lot numbers of reagents are to be recorded on the Bio-Rad D-100 Diary Worksheet. Refer to the QMP for reagent labeling criteria.
2. Analyzer should be in STANDBY or SLEEPING mode prior to beginning analysis.
3. Record the number of injections on the analytical cartridge (column).
4. Record the number of injections on the prefilter.

ii. Sample preparation
1. Specimen Preparation
a. No sample preparation is required. Mixing the samples tubes before loading is not necessary.
b. If the height of the sample (blood) in the tube appears to be ≤ 1 cm (≤ 500 µL), then the samples may need to be prediluted 1:300 prior to analysis:
i. Before pipetting, thoroughly mix the sample by gently inverting the tube.
ii. To predilute, pipet 1.5 mL of Sample Diluent into a labeled 1.5 mL microvial, followed by 5 µL of the whole blood sample.
iii. Cap the sample and mix thoroughly.

2. For Controls, Calibrators, and specimens requiring predilution use a Hamilton Autodilutor to hemolyze and dilute an aliquot of each following this procedure:
    a. On the Auto dilutor, load the program for the D-100.
    b. Place the supply tubing from the Autodilutor into a bottle of Bio-Rad D-100 Diluent.
    c. While pointing the probe into a small beaker, cycle the Autodilutor by pressing the button on the probe around 4 or 5 times to assure that only Bio-Rad D-100 Diluent is in the lines.
    d. Wet a square of gauze with distilled water and wipe the tip of the probe.
    e. Insert the tip into the whole blood specimen and press the button on the probe to draw up the appropriate amount of blood into the tubing.
    f. Insert the tip into a microvial and press the button again to dispense the sample and reagent into the microvial.
    g. Wipe the outside of the tip again the wetted gauze.
    h. Repeat steps i -iii for all controls and for specimens not suitable for direct sampling by the instrument from the primary tube.
3. Loading the Instrument
    a. Controls are performed at the beginning of the run, in positions 1 and 2 of the first rack loaded onto the instrument. Controls are also performed after 50 samples and at the end of the run.
    b. Samples are loaded onto the instrument by placing each capped sample tube in a rack in numerical order following the beginning controls and placing the rack onto the right side of the instrument.
4. Operation of the Bio-Rad D-100 system
    a. Press the Run button.
    b. Monitor the instrument while it runs the samples by looking out for any alerts or warnings that will be announced by beeping and displaying yellow or red on the screen.
    c. When no more racks are on the right side of the instrument, the instrument will end the run and enter the Standby state. The instrument will print a list of results from the run.

d. Refer to the attached instrument's manual for the D-100 Start-up, Operation, Maintenance, and Shut down procedure.
    i. Please note: Instrument Shut Down should be performed before the end of the workday, before the weekend and before any major holiday. At the end of every month, the injection count for each HbA1c test analyzed (for that month), should be retrieved and submitted to a supervisor and/or delegate.
e. Guidelines for the interpretation of results
    i. The D-100 must pass calibration prior to testing patient samples.
    ii. Quality control values must be within acceptable range.
    iii. The total area of each analysis must range from 50,000 - 350,000 units. Results should not be reported if the total area is outside of this range.
    iv. The HbA1c and A0 peaks must be correctly identified.
    v. HbA1c results < 4% and > 14% should be repeated for verification.
    vi. Any sample with >15 %HbA1c, per IFU, should be suspected of having a hemoglobin variant. These samples should be reflexed to the boronate affinity method.
    vii. Any routine HbA1c patient care sample with a combined area of ≥ 50% in the E, D, S, and/or C windows should be suspected of having a homozygous or double-heterozygous variant, or a variant-ß-thalassemia phenotype. Samples with HbF > 30 % are unreportable. The HbA1c result for these samples should not be reported. The result should be reported as "Unreportable" with the added comment, "Presumptive variant or elevated HbF > 30 % consistent with reduced red blood cell lifespan was detected, so the HbA1c test should not be used for this patient. An alternative test to measure glycemia is fructosamine/ glycated serum protein or glycated albumin."
    viii. Common hemoglobin variants such as heterozygous HbS, heterozygous HbC, heterozygous HbD, and heterozygous HbE do not interfere with this method. The results should be reported with the comment, "An abnormal peak consistent with a hemoglobin variant was detected." Other Hb variants have not

been evaluated by the D-100 method and should be reflexed to the boronate affinity method.

ix. Whole blood specimens that have been stored or shipped outside of manufacturer guidelines may exhibit an increase in the P3 peak area. In all cases, all components of the HbA (e.g., P3, Unknown) are appropriately included in the total area to accurately determine the relative percent of HbA1c. Any sample with an "Unknown" or "P3 peak" ≥ 10% should be suspected of having a hemoglobin variant and should be reflexed to the boronate affinity method.

x. HbA1c results less than 4.0% are reported with the comment: "Falsely low HbA1c results may be observed in patients with clinical conditions that shorten erythrocyte life span or decrease mean erythrocyte age. HbA1c may not accurately reflect glycemic control when clinical conditions that affect erythrocyte survival are present. Fructosamine or glycated albumin may be used as an alternate measurement of glycemic control."

xi. While the instrument possesses advanced detection abilities to flag inappropriate chromatograms, the operator should also visually inspect each chromatogram before processing the results.

xii. For samples where abnormal chromatograms are observed (for example in Fig. 1 below; gap seen between the shaded portion and the A1c peak line), samples should be manually diluted and repeated on the D-100.These should also be reflexed to the boronate affinity method. For additional verification/guidance, call technical support at 1-800-224-6723, press # 2 to ask for a technical support.

f. Reporting Results (Procedure: To be performed only by the testing personnel.)

i. When the analytical run is completed, verify QC is acceptable, ensure all specimens are properly identified, and that all specimens are acceptable for reporting by this method. Unacceptable results, specimens that require reflexing, or need further investigation, should be clearly identified.

ii. In the A1c Diary Log database, enter the information from the run into the appropriate spots and print the diary sheet.

iii. Within the D-100 software, touch the "Results" tab to bring up all the results from the day. Utilize the

filter function to display only the samples from the current run.

iv. Touch the checkbox for any and all results that should not be reported. Once they have all been selected, press the "Reject" button.

v. Touch the checkbox for any and all results that should be reported. Once they have all been selected, press the "Release" button.

vi. 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.

g. Reporting Format: Results are expressed as %HbA1c aligned to NGSP units and are rounded to one decimal place.

9. REPORTABLE RANGE OF RESULTS

3.5 to 20.0% HbA1c.

HbA1c results less than 3.5 % are reported as “< 3.5”, and HbA1c results greater than 20.0 % are reported as “> 20.0”

10. QUALITY CONTROL (QC) PROCEDURES

a. Predilute each in-house calibrator 1:300 using the Hamilton Autodilutor to dilute 5 µL of whole blood with 1.5 ml sample diluent.

b. Predilute each in-house control 1:300, using the Hamilton Auto dilutor and an additional microvial containing 1.5 mL sample diluent.

c. Frequency

i. Routine Quality Control Testing – Normal and Abnormal (elevated) controls are run at the beginning of a run, every 50 samples, and at the end of a run (per batch).

ii. Controls should also be analyzed after calibration, after a PM (and after major instrument maintenance), after a change of a critical instrument component, or with software changes (as appropriate).

iii. Sample repeats — Five percent of specimens are randomly selected and reanalyzed in same run. If the difference in %HbA1c between the duplicate is greater than 6% (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 reviewed daily by a supervisor, delegate, or trained tech.

iv. Inter-instrument QC; For Research NGSP QA Purposes Only - Comparison between the D-100 instruments: NGSP monitoring specimens are analyzed each month on each D-100 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).

v. For Research NGSP QA Purposes Only - Comparison between D-100s and the boronate affinity methods (reflexed methods). GHBQC procedure is followed for the comparison of results (n = 30 – 50 per month) between the D-100s HPLC HbA1c method and the boronate affinity reflex HPLC method. Criteria For Pass/Fail: Bland/Altman: +/-0.70, Y at X:+/-0.30, Syx: 0.35. Results are reviewed monthly by the Lab Director.

d. Mean and Ranges:
    1. The assigned values for both the low and high pooled controls were assigned by analyzing once at the beginning of a run and once at the end of a run for at least 10 analytical batches on 10 different days for a minimum of 20 runs.
    2. Quality control limits are established by calculating the 95% (2 SD) and 99% (3 SD) confidence limits for both the daily means and daily ranges for each control.
    3. Refer to the Quality Control binder for additional details.

e. Tolerance limits
    1. The system is declared out of control if any of the following conditions occurs.
        a. The mean from a single run for a single control falls outside the 99% confidence limits (3SD).
        b. The means from a single run for both controls fall outside the 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 1 SD of the established mean are not counted towards this trend.
        d. The range from a single run for a single control fall above the 99% confidence limits.
        e. The ranges from a single run for both controls falls above the 95% confidence limits.
        f. The ranges from eight successive runs for a single control fall above the mean line.

f. Levey-Jennings Plots – Monthly
    1. Mean chart - Plots the mean values for each control in the run and compares them to the upper and lower two and three standard deviation limits as well as the mean.
    2. Range chart - Plots the range of values (maximum value / minimum values) for each control in the run and compares them to the mean, and the upper and lower two and three standard deviations limits.

3. The Laboratory Director or delegate reviews these charts on a monthly basis.

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

1. If either the instrument rejects the calibration or the controls fall outside acceptable criteria, the instrument should undergo troubleshooting to determine the potential causes prior to recalibration. Testing should not be performed until the calibration results are considered acceptable.
2. If a run is declared "out of control", all samples from that run 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 issue, troubleshoot accordingly by performing a calibration and/or pulling new QC. Refer to the Bio-Rad D-100 Operator's Manual for additional troubleshooting guidelines. All troubleshooting activities, including those involving rejected runs, should be documented.
3. Results are not released until the quality control results are acceptable according to the established QC ranges and tolerance limits.

12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS

a. Limitations of the Procedure
   i. Sample Dilution
      1. The required total area range for the D-100 HbA1c test is 50,000 - 350,000 units
      2. If the sample is outside the expected range, manually predilute the sample following the Specimen Preparation guidelines. If the sample area is still outside of the expected range, the sample should be diluted in a manner that can be expected to either increase or decrease the total area. This means that packed red blood cells can be diluted to increase the total area, or a smaller amount of mixed whole blood can be diluted to decrease the total area.
   ii. Special Considerations
      1. The HbA1c test is not intended for analysis of samples collected from newborns.
      2. The HbA1c test should not be used to replace glucose testing in pediatric patients, pregnant women, or patients with Type 1 Diabetes.

3. In cases of rapidly evolving Type 1 diabetes, the increase of HbA1c values might be delayed compared to the acute increase in glucose concentrations. In these conditions, diabetes mellitus must be diagnosed based on plasma glucose concentration and/or the typical clinical symptoms.
4. The HbA1c test should not be used to diagnose diabetes during pregnancy or to diagnose gestational diabetes. HbA_{1c} reflects the average blood glucose levels over the preceding 3 months (the average life of a red blood cell), and therefore may be falsely low during pregnancy or any other condition associated with recent onset of hyperglycemia and/or decreased red cell survival.
5. The HbA1c test should not be used to diagnose diabetes in patients with the following conditions:
    a. Any condition that alters the life span of the red blood cells, including recent blood loss, transfusion, significant iron deficiency, and hemolytic anemia (including hereditary spherocytosis) or other hemolytic diseases, hemoglobinopathies and thalassemias, as the altered red blood cell turnover interferes with the relationship between mean blood glucose and HbA_{1c} values.
    b. Malignancies or severe chronic hepatic or renal disease.

b. Interference
    i. Hemoglobin F concentration up to 30 % do not interfere with this assay, any sample with HbF > 5% should be suspected of having a hemoglobinopathy.
    ii. β-thalassemia trait, as indicated by the increased HbA2 concentration, does not interfere with this test.
    iii. Labile A1c, as indicated by glucose concentrations up to 1200 mg/dL, does not interfere with this test.
    iv. At physiologically occurring concentrations, there is no interference from carbamylated hemoglobin or acetylated hemoglobin.
    v. Common drugs at therapeutic concentrations do not interfere with this test.
    vi. Refer to the Operator's manual for additional details.

13. REFERENCE RANGES (NORMAL VALUES)

a. Reference Ranges
    i. Reference Range: 4 – 5.6 %
    ii. Interpretation Information: Hemoglobin A1C (HbA1c) American Diabetes Association criteria:

≤ 5.6%	Normal
5.7 to 6.4 %	Prediabetes
≥ 6.5%	Diabetes

Repeat hemoglobin A1c testing or follow-up with an alternative test such as the 2-hour OGTT is required prior to the diagnosis of diabetes.

Prediabetes: Patient counseling and commitment to a course of lifestyle modification is recommended with follow-up testing 3-6 months later.

Diabetes mellitus: HbA1c correlates highly with average daily glycemia over the preceding 60–90-day period. While 'good control' is generally considered to be HbA1c < 7.0%, individual factors influence HbA1c goals and attained results, which providers should take into account when using HbA1c in patient management and counseling. In particular, HbA1c does not reliably capture frequency and severity of treatment-related hypoglycemia, which may obligate relaxation of HbA1c goals for the patient.
Note: Any condition that shortens erythrocyte survival or decreases mean erythrocyte age will lower HbA1c results regardless of the assay method. HbA1c results after blood transfusion should be interpreted with caution.

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

14. CRITICAL CALL RESULTS ("PANIC VALUES")

a. 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.

15. SPECIMEN STORAGE AND HANDLING DURING TESTING

Refer to section the specimen collection, storage, and handling procedures; criteria for specimen rejection listed above.

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

N/A

17. TEST RESULT REPORTING SYSTEM; PROTOCOCOL 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 upon receipt in the Weekly Incoming Specimen Log. Samples received are then verified against the Sample Manifest. Refer to Section 3 above for additional details. Specimens are stored under frozen conditions, at -70^{o}C or colder after analysis. Specimen locations are recorded according to sequential DDL accession number and box number and discarded after a minimum of one year.

19. SUMMARY STATISTICS AND QC GRAPHS

Please see following page.

August 2021-August 2023 Summary Statistics and QC Chart
LBXGH (Glycohemoglobin (%))

Lot	n	Start Date	End Date	mean	Standard Deviation	Coefficient of Variation
LBCHGH1_WB30	94	28SEP21	25JAN23	10.41	0.05	0.5
LBCHGH2_WB30	53	28SEP21	25JAN23	10.19	0.12	1.1
LBCHGH3_WB30	58	28SEP21	07JUN23	10.18	0.12	1.2
LBCHGH4_WB30	37	11JAN23	23AUG23	10.43	0.14	1.3
LBCHGL1_WB29	11	28SEP21	24NOV21	5.03	0.03	0.6
LBCHGL1_WB31	83	01DEC21	25JAN23	5.20	0.03	0.5
LBCHGL2_WB29	11	28SEP21	29NOV21	5.14	0.06	1.2
LBCHGL2_WB31	42	01DEC21	25JAN23	5.24	0.05	1.0
LBCHGL3_WB29	11	28SEP21	29NOV21	5.14	0.06	1.2
LBCHGL3_WB31	47	01DEC21	07JUN23	5.24	0.06	1.1
LBCHGL4_WB31	37	11JAN23	23AUG23	5.17	0.05	0.9