GLYCABEN can objectively assess ambient blood glucose providing the physician with an unbiased indication of the efficacy of the prescribed therapy. This allows the physician to monitor and adjust the anti-diabetic therapy in a clinically relevant time frame. Continuous glycemic control is critical for the prevention of diabetic complications such as retinopathy, neuropathy, and nephropathy. GLYCABEN is especially useful for patients with Type I and Type II diabetes and for pregnant diabetic women where optimum control is essential.

**GLYCABEN ASSAY FLOW CHART**

### GLYCABEN

**GLYCABEN**

**Glycated Albumin ELISA**

**INTENDED USE**

GLYCABEN is an enzyme-linked immuno-sorbent assay (ELISA) for the detection and quantitation of glycated albumin in human plasma. It is intended for in-vitro use as an aid in monitoring short-term changes in integrated glycemic control. With a single random sample, GLYCABEN provides a simple, quantitative and reliable measurement of glycated albumin. Such information is analogous to that offered by glycohemoglobin measurements, except that the protein being measured is glycated albumin, which has a shorter half-life (17 days) than does hemoglobin (120 days). It is also analogous to that offered by fructosamine assays, except that it specifically measures a single glycated protein of known residence time rather than non-specifically measuring multiple glycated proteins.

**MEDICAL BACKGROUND**

The measurement of glycated hemoglobins (GHb) has been accepted for the assessment of long-term glycemic control. A more recent innovation is the estimation of glycated plasma proteins by fructosamine measurement (15,16) which, because of the more rapid turnover of these circulating proteins compared with hemoglobin, provides an assessment of shorter-term diabetic control. Circulating plasma proteins are subject to non-enzymatic glycation in the same manner as is the hemoglobin in red cells. Glycation is a slow, continuous and irreversible reaction that is dependent on the glucose concentrations to which the protein is exposed (2). Albumin is the most abundant of the circulating plasma proteins. It has a half-life of about 17 days, much shorter than the 120-day life of the average red cell. As with glycohemoglobin, the amount of glycated albumin is known to increase in patients with poorly controlled diabetes.

The amount of glycated albumin can increase despite normal levels of glycohemoglobin, consistent with the concept that the shorter circulating half-life of albumin relative to that of the erythrocyte reflects blood glucose concentrations over a shorter retrospective period than does glycohemoglobin (5-7,13). Similarly, after the appropriate treatment of a hyperglycemic episode, glycated albumin levels begin to fall before a diminution in glycohemoglobin levels is detectable (8,9,13). Measurement of glycated albumin will confidently reflect average blood glucose levels of the past 2-3 weeks while GHb values indicate control over a 2-3 month period (2,14,17,18). Fructosamine measurement, while also claiming a measurement of glycemic control in the past 2-3 weeks, measures the reducing activity of many different plasma protein types, varying both in concentrations and in circulating half-lives (12,19,21-24).

GLYCABEN can objectively assess ambient blood glucose concentrations over an integrated intermediate time period (2-3 weeks), independent of GHb values in the patient’s cooperation, providing the physician with an unbiased indication of the efficacy of the prescribed therapy. This allows the physician to monitor and adjust the anti-diabetic therapy in a clinically relevant time frame. Continuous glycemic control is critical for the prevention of diabetic complications such as retinopathy, neuropathy, and nephropathy. GLYCABEN is especially useful for patients with Type I and Type II diabetes and for pregnant diabetic women where optimum control is essential.

**PRINCIPLE**

GLYCABEN is a direct non-radiolabel enzyme-linked immunoassay in which glycated albumin in human plasma binds to an immobilized monoclonal antibody that specifically recognizes the glycated moieties on human albumin (3,4). After incubation for a fixed time, an enzyme-conjugated polyclonal antibody is added against human albumin is added. A chromogenic substrate is then added. After the reaction is stopped, the intensity of the color is read in an ELISA reader at 450 nm. The concentration of glycated albumin in the patient sample is read from a calibration curve. The amount of glycated albumin can be expressed as absolute concentration (mg/ml) or as a relative %, determined by dividing the glycated albumin in the sample by the total albumin in the sample. The total albumin in the sample can be determined by using the Bromcresol Green (BCG) Albumin Determination which involves the dye-binding properties of Albumin and is read colorimetrically at 630nm (10,11,20). When albumin binds Bromcresol Green at pH 4.2, the absorbance of the solution increases in direct proportion to the albumin concentration. Quantitation of the plasma protein is calculated from a calibration curve. Standard calibrators and controls are included with the kit and are used each time the assay is performed.

**KIT COMPONENTS**

1. Product Insert
2. Specifications Sheet
3. Glycated Albumin Test Plate: One 96-well plate coated with anti-glycated albumin MAb.
5. Glycated Albumin Neutralizing Buffer: One bottle containing 15 mL of Neutralizing Buffer to neutralize the sample extraction. Contains Sodium Hydroxide, Buffer Salts, Preservatives. Preserves the integrity of the sample extraction. Contains charcoal, buffer salts and preservative.
6. Glycated Albumin Diluent: One bottle containing 10 mL of diluent used for the preparation of standard dilutions for glycated albumin determination. Contains casein, buffer salts and preservative.
7. Glycated Albumin GA Standard: One vial containing glycated albumin standard. This preparation is serially diluted to produce a standard curve. The value of the standard is included on the specifications sheet, and is expressed in mg/mL. Contains human glycated albumin preparation with no preservative. This should be stored at -70°C.
8. Glycated Albumin Assay Control: One vial containing a glycated albumin preparation found to give an intermediate value in the assay. The value is included on the specifications sheet. Contains a mixture of human glycated and non-glycated albumin in buffer salts with no preservatives. This should be stored at -70°C.
9. Glycated Albumin Reaction Buffer: One vial containing 15 mL of Reaction Buffer. Contains buffer salts and preservative.
10. Anti-HSA –HRP Conjugate: One bottle containing 12 mL of anti-human HRP conjugate is supplied in ready to use form. Includes a conjugate prepared in buffer salts, fetal bovine serum and preservative.
11. TMB Color Developer: One bottle containing 12 mL of TMB HRP substrate (TMB in aqueous buffer).
12. Acid Color Stopper: One bottle containing 12 mL of 2 N Sulfuric Acid.
13. Total Albumin Plate: One 96-well plate (uncoated).
14. Total Albumin Standard: One vial containing hAlbumin supplied at 100 mg Albumin/mL. Contains human albumin, buffer salts and preservative.
15. Total Albumin Reagent: Two bottles containing Brom cresol Green (BCG) in buffer salts.

**MATERIALS REQUIRED BUT NOT SUPPLIED**

1. Deionized water
2. 1.7 mL microfuge tubes for sample extraction and standard or control dilution.
3. ELISA plate reader capable of measuring absorbance at 450 nm and at 630 nm
4. Micropipets that accurately deliver 0.01, 0.05, 0.1, 0.15, 0.2 0.25 mL.
5. Multichannel pipettor(s) capable of delivering 100 uL.
6. Incubation Chamber: A plastic container that is large enough to hold the plate and that has a tight fitting cover. It should be assembled with a water moistened paper towel. Complete all incubations at room temperature in this chamber.
7. Microplate Washer
8. Microtube shaker
9. Microscope
10. Wash Buffer: EIA Wash Buffer is recommended. It may be purchased as a 10X concentrate from Exocell, or prepared as a 1X wash buffer of the following composition:

   **1x Wash Buffer:**
   - 0.15 M NaCl
   - 0.01 M Tris(hydroxymethyl)aminomethane (pH 7.4 adjusted with NaOH)
   - 0.05 % Tween 20 (v/v)
   - 0.05 % Proclin 300 (v/v) Preservative (Supoelco, Inc.)

**STORAGE and STABILITY**
The kit components and reagents should be stored under refrigeration, 2°C-8°C. Glycated Standard and Assay Control should be stored frozen at -70°C upon receipt. Properly stored kits and reagents are stable until the expiration date stated in the specifications sheet.

**SPECIMEN COLLECTION AND STORAGE**

Test specimens consist of plasma aseptically drawn by venipuncture using cloting or EDTA anticoagulant. Heparin is to be avoided as anticoagulant. Plasma should be separated from red blood cells as soon as possible to avoid hemolysis. Plasma samples may be stored for 7 days at 4°C or 3 months at -70°C. Specimens should only be frozen and thawed one time. Samples should be allowed to come to room temperature. They should be vortexed briefly, and allowed to rest on the bench top for 15 minutes before charcoal extraction and analysis (to allow particulates, if present, to settle).

**WARNINGS AND PRECAUTIONS**

1. The standards and controls in this kit contain material of human origin. They have been tested for the presence of hepatitis B antigen and for anti-HIV antibodies, but no test can guarantee that the causative agents for hepatitis and AIDS are absent. Therefore, the materials should be viewed as potential biohazards, and should be handled by skilled laboratory personnel.
2. Patient samples should be considered as potential sources of infection, and should be handled by skilled personnel.
3. Avoid mouth pipetting, or eye and skin contact.
4. Do not re-use plates.
5. Avoid using reagents beyond the expiration date (see specifications sheet).
6. Do not subject samples to freeze/thaw cycles more than once.

**LIMITATIONS**

1. Avoid microbial contamination of all reagents and samples, as incorrect assay values may result.
2. Do not use samples with visible hemolysis; every 100 mg hemoglobin/ dl increases the apparent albumin content by an equivalent amount (10).
3. Avoid samples with elevated levels of hemoglobin, bilirubin or lipids. Hemoglobin and lipids may affect the analysis of glycated albumin in GLYCABEN and the BCG-albumin binding assay, producing an incorrect estimation of albumin concentrations (1). This may produce a false low % Glycated Albumin result.
4. Avoid particulates, if present, in the sample.
5. All extractions should be performed overnight with continuous mixing at room temperature. Dilutions should be performed on the same day as the assay.
6. Ampicillin interferes with the BCG assay (1).
7. Phosphate and azide may interfere with the determination of glycated albumin in GLYCABEN, and should be avoided.
8. Avoid hepavin as an anticoagulant for specimen collection.
9. The performance characteristics of serum (rather than plasma) in GLYCABEN have not been fully established. Therefore, the use of serum is not recommended.
10. Performance characteristics of this test to screen for Diabetes Mellitus have not been established.

**Procedure:** Please read this procedure fully and carefully before proceeding.

**GLYCATED ALBUMIN DETERMINATION**

1. **Assay preparation:**
   a. Prepare wash buffer, and adjust pH to 6.8 as required.
   b. Add 250 uL of Glycated Albumin Reaction Buffer to all extracted plasma samples.
   c. Prepare an incubation chamber for the plate.
   d. Add a microtiter plate containing a charcoal mixture to each tube. Be sure that the charcoal mixture is in solution and is not allowed to settle.
   e. Vortex charcoal- plasma mixture for 5 seconds.
   f. Mix overnight at room temperature either by shaking of end-over-end.
   g. After overnight incubation, centrifuge in microfuge at 14K for 10 minutes.
   h. Prepare and label sufficient microtube tubes for extracted plasma samples.
   i. Being careful not to disturb the charcoal pellet, recover 100 uL of the supernatant into a fresh microfuge tube. This contains the albumin portion of the Reagents preparation.

2. **Charcoal Extraction of Plasma Samples:**
   a. Allow Glycated Albumin Charcoal Reagent to come to room temperature.
   b. Prepare and label sufficient microtube tubes for plasma samples to be tested.
   c. Transfer 100 uL of plasma sample to the appropriately labeled tube.
   d. Vigorously mix or vortex the Charcoal Extraction Buffer for 5 seconds.
   e. Add 100 uL Charcoal Extraction Buffer to each tube. Be sure that the charcoal
   f. Mix overnight at room temperature either by shaking of end-over-end.
   g. After overnight incubation, centrifuge in microfuge at 14K for 10 minutes.
   h. Prepare and label sufficient microtube tubes for extracted plasma samples.
   i. Being careful not to disturb the charcoal pellet, recover 100 uL of the supernatant into a fresh microfuge tube. This contains the albumin portion of the Reagents preparation.

4. **Prepare Glycated Albumin Standard Curve:**
   a. The undiluted Glycated Albumin Standard is the stock Glycated Albumin Standard.
   b. Prepare 5 microtube tubes by labeling them 1, 2, 3, 4 and 5.
   c. Add 250 uL of Glycaben Diluent to tube 1, 2, 3, 4 and 5.
   d. Transfer 250 uL of stock Glycated Albumin Standard to tube 1 and mix five times in the tube by aspirating/expelling the fluid. Avoid foaming. This is a 1:2 dilution of the stock Glycated Albumin Standard.
   e. Transfer 250 uL from tube 1 to tube 2, and mix as described in e. above. This is a 1:4 dilution.
   f. Transfer 250 uL from tube 2 to tube 3, and mix as described in e. above. This is a 1:8 dilution.
   g. Transfer 250 uL from tube 3 to tube 4, and mix as described in e. above. This is a 1:16 dilution.
   h. Transfer 250 uL from tube 4 to tube 5, and mix as described in e. above. This is a 1:32 dilution.

5. **Neutralization of Extracted Glycated Plasma Samples:**
   a. Add 100 uL Glycated Albumin Neutralizing Buffer to all extracted plasma containing tubes.
   b. Vortex briefly.
   c. Immediately before Glycated Albumin Primary Incubation or Total Albumin determination, centrifuge in microfuge at 14K for 1 minute.

6. **Primary Incubation:**
   a. Wash the Glycated Albumin Assay Plate 8 times with EIA Wash buffer: A single wash is completed by aspirating off the fluid within the wells, and adding wash buffer to the top.
   b. Aspirate off the fluid from the final wash, and invert the plate on absorbent paper, tapping gently, to remove residual fluids.
   c. Use a clean, dry pipette tip for each addition as detailed below, do not pre-wet tips.
   d. Add 50 uL Glycated Albumin Reaction Buffer to all wells.
   e. Add 50 uL of Glycated Albumin Diluent to wells A1, 2. These are the negative control or &quot;Blank Wells.”
   f. Add Standards:
      i. Add 50 uL of Glycated Standard from tube 1 to wells B1, 2.
      ii. Add 50 uL of Glycated Standard from tube 2 to wells C1, 2.
      iii. Add 50uL of Glycated Standard from tube 3 to wells D1, 2.
      iv. Add 50 uL of Glycated Standard from tube 4 to wells E1, 2.
      v. Add 50 uL of Glycated Standard from tube 5 to wells F1, 2.
      f. Add 50 uL of Assay Control to wells G1, 2.
9. Calculation of Glycated Albumin Results: Regression analysis:
   a. Prepare a plot of standard dilutions with the glycated albumin concentration vs. absorbance. The data that fall into the linear portion of the standard curve constitute the usable portion of the curve.
   b. Subject these data to regression analysis to yield a mathematical model of the form:
      \[ A_{50} = m \cdot \text{glycated albumin} + b \]
   c. Determine the estimated glycated albumin for each experimental sample.
   d. Multiply appropriately for the dilution.
   e. A sample with a value above the range of the standard curve may need to be repeated at a greater dilution.

TOTAL ALBUMIN DETERMINATION

1. Reagent Preparation
   a. Allow all reagents (Total Albumin Standard, BCG Reagent) and patient samples to come to room temperature.
   b. Vortex neutralized charcoal extracted patient samples.
   c. Centrifuge in microfuge at 14K for 10 minutes.
   d. Prepare a detailed plate map for the total albumin analysis.

2. Prepare Serial Dilutions of Total Albumin Standard
   a. Prepare three microfuge tubes by see specifications sheet.
   b. Dispense 50 uL of fluid from tube 1 to tube 2, and mix as described.
   c. Transfer 50 uL of fluid from tube 2 to tube 3, and mix as described.
   d. Continue for the remaining plasma samples.
   e. Add 200 uL of Total Albumin Reagent (BCG) to each of the wells.
   f. Incubate for 5 minutes.
   i. Determine the absorbance at 630 nm, using the negative control well A1 as a blank.

4. Calculation of Total Albumin Results: Graphic Analysis
   a. Secure some linear graph paper.
   b. Plot total albumin concentration (see specifications sheet) on the X-axis and Absorbance at 630 nm on the Y-axis.
   c. Draw a line through the data.
   d. Estimate the sample concentrations by noting where the absorbances intersect the line, and reading off the concentration.
e. All concentrations should be expressed in mg/mL.

5. Calculation of Total Albumin Results: Regression Analysis
   a. Prepare a graph as discussed above.
   b. Compute sample concentrations using a least-squares linear regression model.

REPORTING RESULTS (Please refer to the enclosed specifications sheet)

Glycated albumin can be reported as absolute concentration (mg/mL) or as percent (%) of total albumin as calculated from the following equation using the values determined for each of the samples:

\[ \% \text{Glycated Albumin}_{\text{sample}} = 100 \% \times \frac{\text{Glycated Albumin}_{\text{sample}}}{\text{Total albumin}_{\text{sample}}} \]

QUALITY CONTROL

Review the specifications sheet, the values obtained for the assay control should be within 20% of the indicated value.

INTERPRETATION OF RESULTS

GLYCAEN is useful in measuring the degree of glycemia over short time frames (2-3 weeks) by quantitating the concentration of glycated albumin in plasma. A glycated albumin concentration greater than the established normal range is an indication of hyperglycemia prevailing during the preceding 2-3 weeks.

EXPECTED VALUES

The mean Glycated Albumin level in 100 non-diabetic samples was 1.5% ± 0.1% (SEM). Glycated Hemoglobin levels were less than 3.5%.

When classified by gender, the mean Glycated Albumin level in 46 non-diabetic males was 1.64% ± 0.14% (SEM). The mean Glycated Albumin level in 45 non-diabetic females was 1.39% ± 0.13% (SEM). For the nine whose sex was not identified, the mean % Glycated Albumin was 1.7% ± 0.3% (SEM).

In separate samples from 41 diabetic subjects, the Glycated Albumin levels ranged from 0.8% to 14.9% of total albumin with a mean of 3.9% ± 0.5% (SEM).

The clinical data are summarized as follows:

<table>
<thead>
<tr>
<th>Sample</th>
<th>Glycated Albumin(%) ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-diabetic males</td>
<td>1.5 ± 0.1%</td>
</tr>
<tr>
<td>Non-diabetic females</td>
<td>1.6 ± 0.1%</td>
</tr>
<tr>
<td>Diabetic males</td>
<td>1.4 ± 0.1%</td>
</tr>
<tr>
<td>Diabetic females</td>
<td>1.7 ± 0.2%</td>
</tr>
<tr>
<td>Normal range</td>
<td>0.8% to 14.9%</td>
</tr>
</tbody>
</table>

Normal values should be determined by each laboratory to conform with the characteristics of the population being tested. There is an overlap between normal and diabetic values. Based on the above information and correlative analysis of glycated hemoglobin levels performed at the


Further technical information and performance characteristics of Glycabin are available on request: EOXCELL (800) 234-3962 (USA only) (215) 557-8071

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