



Rat Glycated hemoglobin A1c (GHbA1c) ELISA Kit

Catalog No. CSB-E08140r

(96 tests)

- This immunoassay kit allows for the in vitro quantitative determination of **rat GHbA1c** concentrations in **cell culture supernates, serum, plasma** and other biological fluids.
- **Expiration date** six months from the date of manufacture
- **FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.**

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INTRODUCTION

Glycosylated (or glycated) hemoglobin (hemoglobin A1c, Hb1c , or HbA1c, A1C) is a form of hemoglobin used primarily to identify the average plasma glucose concentration over prolonged periods of time. It is formed in a non-enzymatic pathway by hemoglobin's normal exposure to high plasma levels of glucose. Glycosylation of hemoglobin has been implicated in nephropathy and retinopathy in diabetes mellitus. Monitoring the HbA1c in type-1 diabetic patients may improve treatment.

In the normal 120-day life span of the red blood cell, glucose molecules join hemoglobin, forming glycated hemoglobin. In individuals with poorly controlled diabetes, increases in the quantities of these glycated hemoglobins are noted. Once a hemoglobin molecule is glycated, it remains that way. A buildup of glycated hemoglobin within the red cell reflects the average level of glucose to which the cell has been exposed during its life cycle. Measuring glycated hemoglobin assesses the effectiveness of therapy by monitoring long-term serum glucose regulation. The HbA1c level is proportional to average blood glucose concentration over the previous four weeks to three months.

Higher levels of HbA1c are found in people with persistently elevated blood sugar, as in diabetes mellitus. While diabetic patient treatment goals vary, many include a target range of HbA1c values. A diabetic person with good glucose control has a HbA1c level that is close to or within the reference range. The International Diabetes Federation and American College of Endocrinology recommend HbA1c values below 6.5%, while American Diabetes Association recommends that the HbA1c be below 7.0% for most patients. A high HbA1c represents poor glucose control. However, a 'good' HbA1c in a patient with diabetes can still be riddled with a history of recent hypoglycemia, or, alternatively, spikes of hyperglycemia. Regular blood glucose monitoring is still the best method for the analysis of overall vascular health with respect to blood sugar control. Often, patients with diabetes mellitus are scolded by their doctors for having a HbA1c which is too low, because a lower A1c would indicate a likelihood of frequent hypoglycemia in the recent past. This is often assessed with blood sugar data and receptions are typically mixed. A balance of long term health (hyperglycemia prevention) versus short term health (hypoglycemia prevention) is always a constant concern for both patients and their doctors. Doctors are especially sensitive about lower level HbA1c's with patients who regularly drive, this being a prime example of a short-term motivation for preventing hypoglycemia. Many diabetics have died behind the wheel as a result of a low blood sugar, especially for the reason that frequent hypoglycemia results in a higher tolerance (ideally the patient is seized with a feeling of panic, an increased heart rate, profuse sweating, etc.) for the condition and some patients may not even consciously realize their blood sugar has dropped to dangerous levels. In addition to acquired tolerance, the use of alcohol and certain drugs (marijuana, for example) can create moderately similar symptoms to those of

hypoglycemia (especially when used in combination) and for this reason the patient may not realize he/she has contracted hypoglycemia.

Persistent elevations in blood sugar (and therefore HbA1c) increase the risk for the long-term vascular complications of diabetes such as coronary disease, heart attack, stroke, heart failure, kidney failure, blindness, erectile dysfunction, neuropathy (loss of sensation, especially in the feet), gangrene, and gastroparesis (slowed emptying of the stomach). Poor blood glucose control also increases the risk of short-term complications of surgery such as poor wound healing.

Lower than expected levels of HbA1c can be seen in people with shortened red blood cell life span, such as with glucose-6-phosphate dehydrogenase deficiency, sickle-cell disease, or any other condition causing premature red blood cell death. Conversely, higher than expected levels can be seen in people with a longer red blood cell life span, such as with Vitamin B12 or folate deficiency.

PRINCIPLE OF THE ASSAY

The microtiter plate provided in this kit has been pre-coated with an antibody specific to GHbA1c. Standards or samples are then added to the appropriate microtiter plate wells with a biotin-conjugated polyclonal antibody preparation specific for GHbA1c and Avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. Then a TMB (3,3',5, 5' tetramethyl-benzidine) substrate solution is added to each well. Only those wells that contain GHbA1c, biotin-conjugated antibody and enzyme-conjugated Avidin will exhibit a change in color. The enzyme-substrate reaction is terminated by the addition of a sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450 nm \pm 2 nm. The concentration of GHbA1c in the samples is then determined by comparing the O.D. of the samples to the standard curve.

DETECTION RANGE

15.6 ng/ml-1000 ng/ml. The standard curve concentrations used for the ELISA's were 1000 ng/ml, 500 ng/ml, 250 ng/ml, 125 ng/ml, 62.5 ng/ml, 31.2 ng/ml, 15.6 ng/ml.

SPECIFICITY

This assay recognizes recombinant and natural rat GHbA1c. No significant cross-reactivity or interference was observed.

SENSITIVITY

The minimum detectable dose of rat GHbA1c is typically less than 3.9 ng/ml.

The sensitivity of this assay, or Lower Limit of Detection (LLD) was defined as the lowest protein concentration that could be differentiated from zero.

MATERIALS PROVIDED

Reagent	Quantity
Assay plate	1
Standard	2
Sample Diluent	1 x 20 ml
Biotin-antibody Diluent	1 x 10 ml
HRP-avidin Diluent	1 x 10 ml
Biotin-antibody	1 x 120 μ l
HRP-avidin	1 x 120 μ l
Wash Buffer	1 x 20 ml (25 \times concentrate)
TMB Substrate	1 x 10 ml
Stop Solution	1 x 10 ml

STORAGE

1. Unopened test kits should be stored at 2-8°C upon receipt and the microtiter plate should be kept in a sealed bag with desiccants to minimize exposure to damp air. The test kit may be used throughout the expiration date of the kit. Refer to the package label for the expiration date.
2. Opened test kits will remain stable until the expiring date shown, provided it is stored as prescribed above.
3. A microtiter plate reader with a bandwidth of 10 nm or less and an optical density range of 0-3 OD or greater at 450nm wavelength is acceptable for use in absorbance measurement.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

1. **Wash Buffer** If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Dilute 30 ml of Wash Buffer Concentrate into deionized or distilled water to prepare 500 ml of Wash Buffer.
2. **Standard** Reconstitute the **Standard** with 1.0 ml of **Sample Diluent**. This reconstitution produces a stock solution of 1000 ng/ml. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making serial dilutions. The undiluted standard serves as the high standard (1000 ng/ml). The **Sample Diluent** serves as the zero standard (0 ng/ml).
3. **Biotin-antibody** Dilute to the working concentration specified on the vial label using **Biotin-antibody Diluent**(1:100), respectively.
4. **HRP-avidin** Dilute to the working concentration specified on the vial label using

HRP-avidin Diluent(1:100), respectively.

Precaution: The Stop Solution provided with this kit is an acid solution. Wear eye, hand, face, and clothing protection when using this material.

OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm.
- Pipettes and pipette tips.
- Deionized or distilled water.
- Squirt bottle, manifold dispenser, or automated microplate washer.

SAMPLE COLLECTION AND STORAGE

- **Cell Culture Supernates** Remove particulates by centrifugation and assay immediately or aliquot and store samples at -20°C . Avoid repeated freeze-thaw cycles.
- **Serum** Use a serum separator tube (SST) and allow samples to clot for 30 minutes before centrifugation for 15 minutes at 1000 x g. Remove serum and assay immediately or aliquot and store samples at -20°C . Avoid repeated freeze-thaw cycles.
- **Plasma** Collect plasma using citrate, EDTA, or heparin as an anticoagulant. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection. Assay immediately or aliquot and store samples at -20°C . Avoid repeated freeze-thaw cycles.

Note: Grossly hemolyzed samples are not suitable for use in this assay.

ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all samples, standards, and controls be assayed in duplicate.

1. Add 100 μl of Standard, Blank, or Sample per well. Cover with the adhesive strip. Incubate for 2 hours at 37 $^{\circ}\text{C}$.
2. Remove the liquid of each well, don't wash.
3. Add 100 μl of **Biotin-antibody** working solution to each well. Incubate for 1 hour at 37 $^{\circ}\text{C}$. **Biotin-antibody** working solution may appear cloudy. Warm to room temperature and mix gently until solution appears uniform.
4. Aspirate each well and wash, repeating the process three times for a total of three washes. Wash by filling each well with Wash Buffer (350 μl) using a squirt bottle, multi-channel pipette, manifold dispenser or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.

5. Add 100µl of **HRP-avidin** working solution to each well. Cover the microtiter plate with a new adhesive strip. Incubate for 1 hours at 37° C.
6. Repeat the aspiration and wash three times as step 4.
7. Add 90µl of **TMB Substrate** to each well. Incubate for 30 minutes at 37°C. Keeping the plate away from drafts and other temperature fluctuations in the dark.
8. Add 50µl of **Stop Solution** to each well. If color change does not appear uniform, gently tap the plate to ensure thorough mixing.
9. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm.

CALCULATION OF RESULTS

Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density. Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the GHbA1c concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data. If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

LIMITATIONS OF THE PROCEDURE

- The kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- It is important that the Calibrator Diluent selected for the standard curve be consistent with the samples being assayed.
- If samples generate values higher than the highest standard, dilute the samples with the appropriate Calibrator Diluent and repeat the assay.
- Any variation in Standard Diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- This assay is designed to eliminate interference by soluble receptors, binding proteins, and other factors present in biological samples. Until all factors have been tested in the Quantikine Immunoassay, the possibility of interference cannot be excluded.

TECHNICAL HINTS

- When mixing or reconstituting protein solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.

- When using an automated plate washer, adding a 30 second soak period following the addition of wash buffer, and/or rotating the plate 180 degrees between wash steps may improve assay precision.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- Substrate Solution should remain colorless until added to the plate. Keep Substrate Solution protected from light. Substrate Solution should change from colorless to gradations of blue.
- Stop Solution should be added to the plate in the same order as the Substrate Solution. The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution. Wells that are green in color indicate that the Stop Solution has not mixed thoroughly with the Substrate Solution.