Innovative RESEARCH Results Driven Research

Human Glucokinase/ Hexokinase-4 ELISA Kit

Introduction

Human Glucokinase (GCK), also known as hexokinase IV or D, is a 50 kDa monomeric protein of 465 amino acids (1-2). It is present in the liver, pancreas, small intestine and brain. It plays important roles in glucose metabolism. In response to rising levels of glucose from eating, GCK activity increases rapidly. It catalyzes the transfer of phosphate from ATP to glucose to form glucose-6-phosphate, which is the first, rate-limiting step of glycogen synthesis and glycolysis. By means of this reaction, it functions as a glucose sensor for insulin secretion in pancreatic β-cells, and regulates glucose and glycogen production in the liver (3). Mutations of the GCK gene are associated with non-insulin-dependent diabetes mellitus (4), persistent hyperinsulinemic hypoglycemia of infancy (5) and maturity-onset diabetes of younger individuals (6). GCK is a drug target for developing anti-type 2 diabetic molecules.

Principal of the Assay

The Human Glucokinase ELISA (Enzyme-Linked Immunosorbent Assay) kit is designed for detection of human Glucokinase in plasma, serum, and cell culture supernatants. This assay employs a quantitative sandwich enzyme immunoassay technique that measures human Glucokinase in less than 4 hours. A polyclonal antibody specific for human Glucokinase has been pre-coated onto a 96-well microplate with removable strips. Glucokinase in standards and samples is sandwiched by the immobilized antibody and the biotinylated polyclonal antibody specific for Glucokinase, which is recognized by a streptavidin-peroxidase conjugate. All unbound material is then washed away and a peroxidase enzyme substrate is added. The color development is stopped and the intensity of the color is measured.

Caution and Warning

- **Prepare all reagents (working diluent buffer, wash buffer, standards, biotinylated-antibody, and SP conjugate) as instructed, prior to running the assay.**
- **Prepare all samples prior to running the assay. The dilution factors for the samples are suggested in this protocol. However, the user should determine the optimal dilution factor.**
- **Spin down the SP conjugate vial and the biotinylated-antibody vial before opening and using contents.**
- This kit is for research use only.
- The kit should not be used beyond the expiration date.
- The Stop Solution is an acid solution

Reagents

- **Human GCK Microplate:** A 96-well polystyrene microplate (12 strips of 8 wells) coated with a polyclonal antibody against human GCK.
- **Sealing Tapes:** Each kit contains 3 pre-cut, pressure-sensitive sealing tapes that can be cut to fit the format of the individual assay.
- **Human GCK Standard:** Human GCK in a buffered protein base (50 ng, lyophilized).
- **Biotinylated GCK Antibody (60x):** A 60-fold concentrated biotinylated polyclonal antibody against GCK (125 µl).
- **MIX Diluent Concentrate (10x)**: A 10-fold concentrated buffered protein base (30 ml).
- **Wash Buffer Concentrate (20x)**: A 20-fold concentrated buffered surfactant (30 ml, 2 bottles).
- **Streptavidin-Peroxidase Conjugate (SP Conjugate):** A 100-fold concentrate (80 µl).
- **Chromogen Substrate**: A ready-to-use stabilized peroxidase chromogen substrate tetramethylbenzidine (8 ml).
- **Stop Solution**: A 0.5 N hydrochloric acid to stop the chromogen substrate reaction (12 ml).

Storage Condition

- Store components of the kit at 2-8^{0}C or -20^{0}C upon arrival up to the expiration date.
- Store SP Conjugate and Biotinylated Antibody at -20^{0}C
- Store Microplate, Diluent Concentrate (10x), Wash Buffer, Stop Solution, and Chromogen Substrate at 2-8^{0}C
- Opened unused microplate wells may be returned to the foil pouch with the desiccant packs. Reseal along zip-seal. May be stored for up to 1 month in a vacuum desiccator.
- Diluent (1x) may be stored for up to 1 month at 2-8^{0}C.
- Store Standard at 2-8^{0}C before reconstituting with Diluent and at -20^{0}C after reconstituting with Diluent.

Other Supplies Required

- Microplate reader capable of measuring absorbance at 450 nm.
- Pipettes (1-20 µl, 20-200 µl, 200-1000µl and multiple channel).
- Deionized or distilled reagent grade water.

Sample Collection, Preparation and Storage

- **Plasma:** Collect plasma using one-tenth volume of 0.1 M sodium citrate as an anticoagulant. Centrifuge samples at 2000 x g for 10 minutes and assay. The undiluted samples can be stored at -20^{0}C or below for up to 3 months. Avoid repeated freeze-thaw cycles. (EDTA or Heparin can also be used as anticoagulant.)
- **Serum:** Samples should be collected into a serum separator tube. After clot formation, centrifuge samples at 2000 x g for 10 minutes. Remove serum and assay. The undiluted samples can be stored at -20^{0}C or below for up to 3 months. Avoid repeated freeze-thaw cycles.
- **Cell Culture Supernatants:** Centrifuge cell culture media at 2000 x g for 10 minutes to remove debris. Collect supernatants and assay. Store the remaining samples at -20^{0}C or below. Avoid repeated freeze-thaw cycles.

Reagent Preparation

- Freshly dilute all reagents and bring all reagents to room temperature before use.

- **MIX Diluent Concentrate (10x):** If crystals have formed in the concentrate, mix gently until the crystals have completely dissolved. Dilute the MIX Diluent 1:10 with reagent grade water. Store for up to 1 month at 2-8^{0}C.
- **Standard Curve:** Reconstitute the 50 ng of GCK Standard with 1 ml of MIX Diluent to generate a solution of 50 ng/ml. Allow the standard to sit for 10 minutes with gentle agitation prior to making dilutions. Prepare duplicate or triplicate standard points by serially diluting the standard solution (50 ng/ml) 1:2 with MIX Diluent to produce 25, 12.5, 6.25, 3.13 and 1.56 ng/ml solutions. MIX Diluent serves as the zero standard (0 ng/ml). Any remaining solution should be frozen at -20^{0}C.

Standard Point	Dilution	[GCK] (ng/ml)
P1	Standard (50 ng/ml)	50.0
P2	1 part P1 + 1 part MIX Diluent	25.0
P3	1 part P2 + 1 part MIX Diluent	12.5
P4	1 part P3 + 1 part MIX Diluent	6.25
P5	1 part P4 + 1 part MIX Diluent	3.13
P6	1 part P5 + 1 part MIX Diluent	1.56
P7	MIX Diluent	0.00

- **Biotin GCK Antibody (60x):** Spin down the antibody briefly and dilute the desired amount of the antibody 1:60 with MIX Diluent. Any remaining solution should be frozen at -20^{0}C.
- **Wash Buffer Concentrate (20x):** Dilute the Wash Buffer Concentrate 1:20 with reagent grade water.
- **SP Conjugate (100x):** Spin down the SP Conjugate briefly and dilute the desired amount of the conjugate 1:100 with MIX Diluent. Any remaining solution should be frozen at -20^{0}C.

Assay Procedure

- Prepare all reagents, working standards and samples as instructed. Bring all reagents to room temperature before use. The assay is performed at room temperature (20-30^{0}C).
- Remove excess microplate strips from the plate frame and return them immediately to the foil pouch with desiccant inside. Reseal the pouch securely to minimize exposure to water vapor and store in a vacuum desiccator.
- Add 50 µl of GCK standard or sample per well. Cover wells with a sealing tape and incubate for two hours. Start the timer after the last sample addition.
- Wash five times with 200 µl of Wash Buffer manually. Invert the plate each time and decant the contents; hit it 4-5 times on absorbent paper towel to completely remove the liquid. If using a machine wash six times with 300 µl of Wash Buffer and then invert the plate, decant the contents; hit it 4-5 times on absorbent paper towel to completely remove the liquid.
- Add 50 µl of Biotinylated GCK Antibody to each well and incubate for one hour.
- Wash the microplate as described above.
- Add 50 µl of Streptavidin-Peroxidase Conjugate to each well and incubate for 30 minutes. Turn on the microplate reader and set up the program in advance.
- Wash the microplate as described above.
- Add 50 µl of Chromogen Substrate per well and incubate for about 20 minutes or till the optimal blue color density develops. Gently tap plate to ensure thorough mixing and break the bubbles in the well with pipette tip.
- Add 50 µl of Stop Solution to each well. The color will change from blue to yellow.
- Read the absorbance on a microplate reader at a wavelength of 450 nm **immediately**. If wavelength correction is available, subtract readings at 570 nm from those at 450 nm to correct optical imperfections. Otherwise, read the plate at 450 nm only. Please note that some

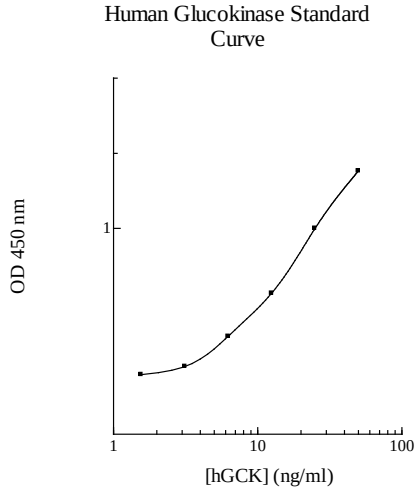
unstable black particles may be generated at high concentration points after stopping the reaction for about 10 minutes, which will reduce the readings.

Data Analysis

- Calculate the mean value of the duplicate or triplicate readings for each standard and sample.
- To generate a standard curve, plot the graph using the standard concentrations on the x-axis and the corresponding mean 450 nm absorbance on the y-axis. The best-fit line can be determined by regression analysis using four-parameter or log-log logistic curve-fit.
- Determine the unknown sample concentration from the Standard Curve and multiply the value by the dilution factor.

Standard Curve

- The curve is provided for illustration only. A standard curve should be generated each time the assay is performed.



Sensitivity and Specificity

- The minimum detectable dose of GCK is typically ~ 1.5 ng/ml.
- Intra-assay and inter-assay coefficients of variation were 5.1% and 7.4% respectively.

Linearity

Sample Dilution	Plasma	Serum
No dilution	84%	89%
1:2	95%	98%
1:4	105%	107%

Recovery

Standard Added Value	1 – 10 ng/ml
Recovery %	85 - 104 %
Average Recovery %	96%

Cross-Reactivity

Species	% Cross Reactivity
Canine	100%
Bovine	5%
Monkey	20%
Mouse	None
Rat	None
Swine	None
Rabbit	None

References

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(3) Matschinsky FM (2009) Nat Rev Drug Discov. 8(5):399-416
(4) Froguel P et al. (1992) Nature. 356(6365):162-164
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(6) Fajans SS et al. (2001) N Engl J Med. 345(13):971-980

Version 1.6