

## Human GAD65 ELISA Kit

Cat #: orb406343 (manual)

Size: 96T

*For Research Use Only. Not For Use in Diagnostic Procedures!*

### Product Features

**Intend Use:** For the quantitative determination of human glutamic acid decarboxylase 65 (GAD65) concentrations in serum, plasma, tissue homogenates.

**Sample Types:** serum, plasma, tissue homogenates

**Detection Range:** 0.156 ng/ml-10 ng/ml

**Sensitivity:** 0.039 ng/ml

**Specificity:** No significant cross-reactivity or interference between human GAD65 and analogues was observed.

### Assay Principle

This assay employs the quantitative sandwich enzyme immunoassay technique. Antibody specific for GAD65 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any GAD65 present is bound by the immobilized antibody. After removing any unbound substances, a biotin-conjugated antibody specific for GAD65 is added to the wells. After washing, avidin conjugated Horseradish Peroxidase (HRP) is added to the wells. Following a wash to remove any unbound avidin-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of GAD65 bound in the initial step. The color development is stopped, and the intensity of the color is measured.

### Kit Components

Reagents	Quantity
Assay plate (12 x 8 coated Microwells)	1 (96 wells)
Standard (Freeze dried)	2
Biotin-antibody (100 x concentrate)	1 x 120 µl
HRP-avidin (100 x concentrate)	1 x 120 µl
Biotin-antibody Diluent	1 x 15 ml
HRP-avidin Diluent	1 x 15 ml
Sample Diluent	1 x 50 ml

Wash Buffer (25 x concentrate)	1 x 20 ml
TMB Substrate	1 x 10 ml
Stop Solution	1 x 10 ml
Adhesive Strip (For 96 wells)	4
Instruction manual	1

### Storage

Unopened kit	Store at 2 - 8°C. Do not use the kit beyond the expiration date.	
Opened kit	Coated assay plate	May be stored for up to 1 month at 2 - 8°C. Try to keep it in a sealed aluminum foil bag and avoid the damp.
	Standard	May be stored for up to 1 month at 2 - 8° C. If don't make recent use, better keep it store at -20°C.
	Biotin-antibody	
	HRP-avidin	
	Biotin-antibody Diluent	May be stored for up to 1 month at 2 - 8°C.
	HRP-avidin Diluent	
	Sample Diluent	
	Wash Buffer	
	TMB Substrate	
	Stop Solution	

### Materials Required but Not Supplied

1. Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm.
2. An incubator which can provide stable incubation conditions up to 37°C±0.5°C.
3. Squirt bottle, manifold dispenser, or automated microplate washer.
4. Absorbent paper for blotting the microtiter plate.
5. 100ml and 500ml graduated cylinders.
6. Deionized or distilled water.
7. Pipettes and pipette tips.
8. Test tubes for dilution.

## Sample Preparation

### Sample Collection and Storage

**Serum:** Use a serum separator tube (SST) and allow samples to clot for two hours at room temperature or overnight at 4°C before centrifugation for 15 minutes at 1000 ×g. Remove serum and assay immediately or aliquot and store samples at -20°C or -80°C. Avoid repeated freeze-thaw cycles.

**Plasma:** Collect plasma using EDTA, or heparin as an anticoagulant. Centrifuge for 15 minutes at 1000 ×g at 2 - 8°C within 30 minutes of collection. Assay immediately or aliquot and store samples at -20°C or -80°C. Avoid repeated freeze-thaw cycles. Centrifuge the sample again after thawing before the assay.

**Tissue Homogenates:** 100mg tissue was rinsed with 1X PBS, homogenized in 1 ml of 1X PBS and stored overnight at -20°C. After two freeze-thaw cycles were performed to break the cell membranes, the homogenates were centrifuged for 5 minutes at 5000 x g, 2 - 8°C. The supernate was removed and assayed immediately. Alternatively, aliquot and store samples at -20°C or -80°C. Centrifuge the sample again after thawing before the assay. Avoid repeated freeze-thaw cycles.

### Notes:

1. We are only responsible for the kit itself, but not for the samples consumed during the assay. The user should calculate the possible amount of the samples used in the whole test. Please reserve sufficient samples in advance.
2. Samples to be used within 5 days may be stored at 2-8°C, otherwise samples must be stored at -20°C (≤1month) or -80°C (≤2months) to avoid loss of bioactivity and contamination.
3. Grossly hemolyzed samples are not suitable for use in this assay.
4. If the samples are not indicated in the manual, a preliminary experiment to determine the validity of the kit is necessary.
5. Please predict the concentration before assaying. If values for these are not within the range of the standard curve, users must determine the optimal sample dilutions for their particular experiments.
6. Tissue or cell extraction samples prepared by chemical lysis buffer may cause unexpected ELISA results due to the impacts of certain chemicals.
7. Owing to the possibility of mismatching between antigen from other resource and antibody used in our kits (e.g., antibody targets conformational epitope rather than linear epitope), some native or recombinant proteins from other manufacturers may not be recognized by our products.
8. Influenced by the factors including cell viability, cell number and also sampling time, samples from cell culture supernatant may not be detected by the kit.
9. Fresh samples without long time storage are recommended for the test. Otherwise, protein degradation and denaturalization may occur in those samples and finally lead to wrong results.

## Reagent Preparation

- Kindly use graduated containers to prepare the reagent. Please don't prepare the reagent directly in the Diluent vials provided in the kit.
- Bring all reagents to room temperature (18-25°C) before use for 30min.
- Prepare fresh standard for each assay. Use within 4 hours and discard after use.
- Making serial dilution in the wells directly is not permitted.

- Please carefully reconstitute Standards according to the instruction and avoid foaming and mix gently until the crystals have completely dissolved. To minimize imprecision caused by pipetting, use small volumes and ensure that pipettors are calibrated. It is recommended to suck more than 10µl for once pipetting.
- Distilled water is recommended to be used to make the preparation for reagents. Contaminated water or container for reagent preparation will influence the detection result.

#### 1. Biotin- antibody (1x) - Centrifuge the vial before opening.

Biotin-antibody requires a 100-fold dilution. A suggested 100-fold dilution is 10 µl of Biotin-antibody + 990 µl of Biotin-antibody Diluent.

#### 2. HRP-avidin (1x) - Centrifuge the vial before opening.

HRP-avidin requires a 100-fold dilution. A suggested 100-fold dilution is 10 µl of HRP-avidin + 990 µl of HRP-avidin Diluent.

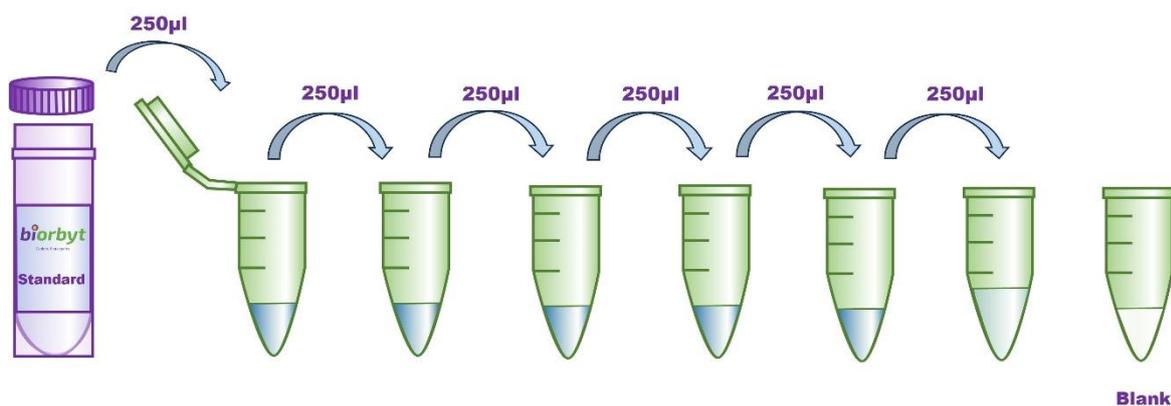
**3. Wash Buffer(1x)-** If crystals have formed in the concentrate, warm up to room temperature and mix gently until the crystals have completely dissolved. Dilute 20 ml of Wash Buffer Concentrate (25 x) into deionized or distilled water to prepare 500 ml of Wash Buffer (1 x).

#### 4. Standard

Centrifuge the standard vial at 6000-10000rpm for 30s.

Reconstitute the **Standard** with 1.0 ml of **Sample Diluent**. Do not substitute other diluents. This reconstitution produces a stock solution of 10 ng/ml. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Pipette 250 µl of **Sample Diluent** into each tube (S0-S6). Use the stock solution to produce a 2-fold dilution series (below). Mix each tube thoroughly before the next transfer. The undiluted Standard serves as the high standard (10 ng/ml). **Sample Diluent** serves as the zero standard (0 ng/ml).



Tube	S7	S6	S5	S4	S3	S2	S1	S0
ng/ml	10	5	2.5	1.25	0.625	0.312	0.156	0

#### Assay procedure

**Bring all reagents and samples to room temperature before use. Centrifuge the sample again after thawing before the assay. It is recommended that all samples and standards be assayed in duplicate.**

1. Prepare all reagents, working standards, and samples as directed in the previous sections.
2. Refer to the Assay Layout Sheet to determine the number of wells to be used and put any remaining wells and the desiccant back into the pouch and seal the ziploc, store unused wells at 4°C.
3. Add 100µl of standard and sample per well. Cover with the adhesive strip provided. Incubate for 2 hours at 37°C. A plate layout is provided to record standards and samples assayed.
4. Remove the liquid of each well, don't wash.
5. Add 100µl of Biotin-antibody (1x) to each well. Cover with a new adhesive strip. Incubate for 1 hour at 37°C. (Biotin-antibody (1x) may appear cloudy. Warm up to room temperature and mix gently until solution appears uniform.)
6. Aspirate each well and wash, repeating the process two times for a total of three washes. Wash by filling each well with Wash Buffer (200µl) using a squirt bottle, multi-channel pipette, manifold dispenser, or autowasher, and let it stand for 2 minutes, 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.
7. Add 100µl of HRP-avidin (1x) to each well. Cover the microtiter plate with a new adhesive strip. Incubate for 1 hour at 37°C.
8. Repeat the aspiration/wash process for five times as in step 6.
9. Add 90µl of TMB Substrate to each well. Incubate for 15-30 minutes at 37°C. Protect from light.
10. Add 50µl of Stop Solution to each well, gently tap the plate to ensure thorough mixing.
11. Determine the optical density of each well within 5 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. Subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

Notes:

1. The final experimental results will be closely related to validity of the products, operation skills of the end users and the experimental environments.
2. Samples or reagents addition: Please use the freshly prepared Standard. Please carefully add samples to wells and mix gently to avoid foaming. Do not touch the well wall as possible. For each step in the procedure, total dispensing time for addition of reagents or samples to the assay plate should not exceed 10 minutes. This will ensure equal elapsed time for each pipetting step, without interruption. Duplication of all standards and specimens, although not required, is recommended. 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.
3. Incubation: To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary. Do not allow wells to sit uncovered for extended periods between incubation steps. Once reagents have been added to the well strips, DO NOT let the strips DRY at any time during the assay. Incubation time and temperature must be observed.
4. Washing: The wash procedure is critical. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Solution by aspirating or decanting and remove any drop of water and fingerprint on the bottom of the plate. Insufficient washing will result in poor precision and falsely elevated absorbance reading. When using an automated plate washer, adding a

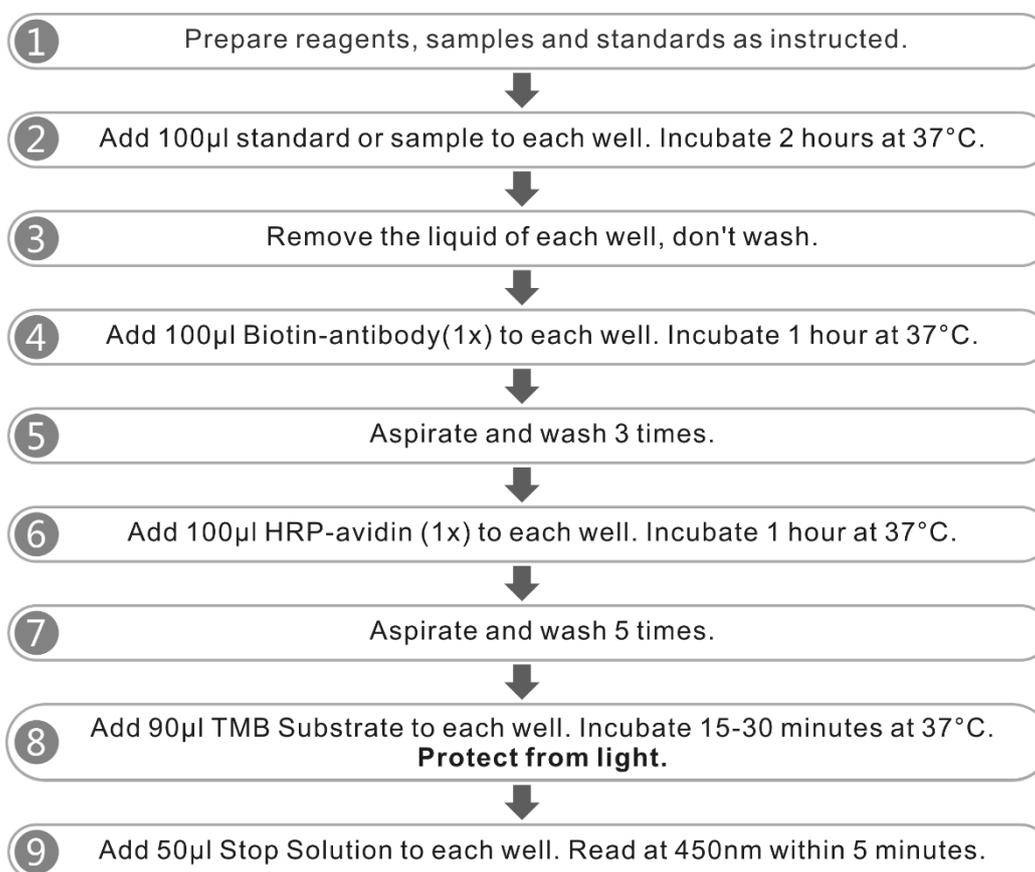
30 second soak period following the addition of wash buffer, and/or rotating the plate 180 degrees between wash steps may improve assay precision.

5. Controlling of reaction time: Observe the change of color after adding TMB Substrate (e.g. observation once every 10 minutes), TMB Substrate should change from colorless or light blue to gradations of blue. If the color is too deep, add Stop Solution in advance to avoid excessively strong reaction which will result in inaccurate absorbance reading.

6. TMB Substrate is easily contaminated. TMB Substrate should remain colorless or light blue until added to the plate. Please protect it from light.

7. Stop Solution should be added to the plate in the same order as the TMB Substrate. 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 TMB Substrate.

## Assay procedure Summary



**\*Please determine whether the sample needs to be diluted or the optimal dilution factor based on preliminary experiment result.**

## Calculation of Results

### Using the professional soft "Curve Expert" to make a standard curve is recommended

Average the duplicate readings for each standard 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 x-axis against the concentration on the y-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the GAD65 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.

## Performance

### Detection Range

0.156 ng/ml-10 ng/ml.

### Sensitivity

The minimum detectable dose of human GAD65 is typically less than 0.039 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. It was determined the mean O.D value of 20 replicates of the zero standard added by their three standard deviations.

### Specificity

This assay has high sensitivity and excellent specificity for detection of human GAD65. No significant cross-reactivity or interference between human GAD65 and analogues was observed.

Note: Limited by current skills and knowledge, it is impossible for us to complete the cross-reactivity detection between human GAD65 and all the analogues, therefore, cross reaction may still exist.

### Precision

#### Intra-assay Precision (Precision within an assay): CV%<8%

Three samples of known concentration were tested twenty times on one plate to assess.

#### Inter-assay Precision (Precision between assays): CV%<10%

Three samples of known concentration were tested in twenty assays to assess.

## ELISA Troubleshooting

### High background/non-specific staining

Description of results	Possible reason	Recommendations and precautions
After stopping the reaction, the entire plate shows a uniform yellow or light color, or the standard curve is linear but the background signal is excessively high.	Yellowing of the entire plate may be caused by incorrect addition of reagents.	Before starting the experiment, verify the components and lot numbers of all reagents to ensure they belong to the same kit. Reagents from different kits or different lot numbers must not be mixed.
	The ELISA plate was not washed sufficiently.	Ensure that the same volume of Wash Solution is added to each microwell during the washing process. After washing, firmly tap the ELISA plate on absorbent paper to remove any residual buffer.
	Incubation time was too long.	Strictly follow the procedures outlined in the manual.
	Streptavidin-HRP contaminated the pipette tip or TMB container, or the positive control contaminated the pre-coated microplate.	Replace pipette tips when dispensing different reagents. Use separate containers when preparing different reagent components, and always use a pipette during handling.
	The concentration of Biotinylated Antibody or Streptavidin-HRP was too high.	Verify that concentration calculations are correct, or perform further dilution if necessary.
	The substrate was exposed to light or contaminated prior to use.	Store reagents in the dark at all times prior to substrate addition.
	Color development time was too long.	Strictly follow the procedures outlined in the manual.
	An incorrect filter was used when reading the absorbance value.	When TMB is used as the substrate, measure absorbance at 450 nm.

### NO color plates

Description of results	Possible reason	Recommendations and precautions
After the color development step, all wells of the ELISA plate are	Components from different reagent sets were mixed.	Carefully read reagent labels when preparing or using them.

colorless, and the positive control is not clearly detectable. s	During plate washing or sample/enzyme addition, the enzyme label was contaminated or inactivated, resulting in loss of its ability to catalyze color development.	Confirm that the container holding the ELISA plate does not contain enzyme inhibitors (such as $\text{NaN}_3$ ), and ensure that the container used to prepare the Wash Solution has been thoroughly cleaned.
	A reagent or procedural step was omitted.	Review the manual carefully and strictly follow the operating procedures.

### Light color

Description of results	Possible reason	Recommendations and precautions
The Standard appears normal, but the sample color is weak.	The sample contains $\text{NaN}_3$ preservative, which inhibits the enzyme reaction.	Samples must not contain $\text{NaN}_3$ .
	The sample being tested may not contain strongly positive material, so the result may be normal.	If there is any doubt about the results, repeat the assay.
The visual result appears normal, but the microplate reader values are low.	An incorrect filter was used for absorbance measurement.	When TMB is used as the substrate, absorbance should be measured at 450 nm.
All wells, including Standards and Samples, show weak color development.	Insufficient incubation time.	Ensure accurate timing using a timer.
	Inadequate color development.	Typically 15–30 minutes.
	Excessive washing, or the dilution ratio of the concentrated Wash Buffer does not meet requirements.	Minimize the impact of washing by diluting the concentrated Wash Buffer and setting the washing time according to the manual. Accurately record the number of washes and the volume used.
	Poor quality distilled water.	The prepared Wash Buffer should be tested to confirm that the pH is neutral.
	During plate washing or sample addition, the enzyme label was contaminated or inactivated, resulting in loss of catalytic activity.	Confirm that the container holding the ELISA plate does not contain enzyme inhibitors (such as $\text{NaN}_3$ ), that the container used to prepare the Wash Buffer has been thoroughly cleaned, and that the purified water used meets the required standards and is not contaminated.
	The kit has expired or was improperly stored.	Use the kit within its expiration date and store it according to the conditions recommended in the manual to avoid contamination.

	Reagents and samples were not equilibrated to room temperature before use.	Allow all reagents and samples to equilibrate at room temperature for approximately 30 minutes before use.
	Insufficient pipetting volume, overly rapid dispensing, excessive liquid remaining on the inner wall of the tip, or unclean tip walls.	Calibrate the pipette properly. Ensure tips are compatible and fit securely, pipette at an appropriate speed, and fully dispense the liquid. Tips should have clean inner walls and be used only once
Poor repeatability.	Incubation temperature was not properly controlled.	Maintain a constant incubation temperature and avoid localized temperature extremes.
	Excess liquid remained on the inner wall of the wells during liquid addition.	When adding liquids, dispense along the lower inner wall of the wells without touching the bottom.
	Reuse of consumables.	Replace pipette tips when drawing different reagents, and use separate containers when preparing different reagent components.
	The bottom of the microwell is scratched or contaminated.	Handle the plate carefully. Avoid touching the well bottoms and clean the bottom of the microplate to remove dirt or fingerprints. Perform technical replicates of the same sample three times, ensuring that at least two values are comparable.
	Cross-contamination during sample addition.	Minimize the risk of cross-contamination during sample addition.
The color development across the plate is uneven and irregular.	Cross-contamination during manual plate washing.	When washing plates manually, discard the first three washes immediately, then allow soaking during subsequent washes to reduce cross-contamination.
	Cross-contamination during plate tapping.	Use appropriate absorbent paper when tapping the plate. Avoid introducing foreign material into the wells, and avoid tapping in the same position repeatedly to reduce cross-contamination.

Description of results	Possible reason	Recommendations and precautions
The color development of the plate is uneven and irregular	The liquid dispensing head of the plate washer is clogged, leading to improper liquid dispensing or excessive residual liquid after aspiration, which results in uneven and irregular color development across the plate.	Unclog the liquid dispensing head to ensure that each well is properly filled with wash solution during plate washing and that minimal residual liquid remains after aspiration.

Incomplete centrifugation of the sample, resulting in coagulation within the reaction wells or interference from sediment or residual cellular components.	Serum and plasma samples should be fully centrifuged at 3000 rpm for more than 6 minutes.
The sample was stored for an excessively long period, leading to contamination.	Samples should be kept fresh or stored at low temperatures to prevent contamination.
Incorrect preparation of the Wash Solution or direct use of the concentrated Wash Solution.	Prepare all reagents strictly according to the manual.

### Declaration

1. FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
2. The kit should not be used beyond the expiration date.
3. Do not mix or substitute reagents with those from other lots or sources.
4. If samples generate values higher than the highest standard, dilute the samples with Sample Diluent and repeat the assay.
5. Any variation in Sample Diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
6. 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 Immunoassay, the possibility of interference cannot be excluded.

### Safety Notes

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