ELISA Kit for Measuring 14-3-3 Gamma

CircuLex 14-3-3 Gamma ELISA Kit

Cat# CY-8082

Intended Use

The MBL Research Product **CircuLex 14-3-3 Gamma ELISA Kit** is used for the quantitative measurement of 14-3-3 Gamma (gamma isoform protein) in CSF (Cerebrospinal fluid), cell lysates in units of concentration.

Individual users should determine appropriate conditions when using other types of samples.

This assay kit is for research use only and not for use in diagnostic or therapeutic procedures.

Storage

- Upon receipt store all components at 4°C.
- Don't expose reagents to excessive light.

Introduction

14-3-3 proteins are a family of conserved regulatory molecules expressed in all eukaryotic cells. There are seven genes, β, γ, ε, σ, ζ, τ and η, that encode 14-3-3s in most mammals. 14-3-3 proteins have the ability to bind phospho-Ser/Thr motifs, as well as phosphorylation-independent interactions, on a multitude of functionally diverse signaling proteins, including kinases, phosphatases, and transmembrane receptors. The family dynamically regulates the activity of target proteins in various signaling pathways that control diverse physiological and pathological processes. More than 100 signaling proteins have been reported as 14-3-3 proteins' targets.

Either an abnormal state of 14-3-3 protein expression or dysregulation of 14-3-3/terget protein interactions contributes to the development of many human diseases. Clinical investigations have demonstrated a correlation between up-regulated 14-3-3 protein levels and poor survival of cancer patients. Several studies have also suggested that 14-3-3 isoforms are differentially regulated in cancer and neurological syndromes. Especially, elevated amounts of the gamma isoform (14-3-3 Gamma) are found in the cerebrospinal fluid (CSF) of Creutzfeldt-Jakob disease (CJD) patients and could be as a specific marker of the disease as well as tau protein level.

Principle of the Assay

The MBL Research Product **CircuLex 14-3-3 Gamma ELISA Kit** employs the quantitative sandwich enzyme immunoassay technique. An antibody specific for 14-3-3 Gamma has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and the immobilized antibody binds any 14-3-3 Gamma present. After washing away any unbound substances, a detection antibody specific for 14-3-3 Gamma is added to the wells, followed by binding with horseradish peroxidase (HRP) conjugated anti-IgG antibody. Following a wash to remove any unbound HRP conjugate, the remaining conjugate is allowed to react with the substrate H_2O_2-tetramethylbenzidine. The reaction is stopped by addition of acidic solution and absorbance of the resulting yellow product is measured at 450 nm. The absorbance is proportional to the concentration of 14-3-3 Gamma. A standard curve is constructed by plotting absorbance values versus the units of 14-3-3 Gamma concentration of calibrators, and units of concentration of unknown samples are determined using this standard curve.

Summary of Procedure

Add 100 µL of 14-3-3 Gamma Standard Solutions and diluted samples to wells
↓ Incubate for 60 minutes at 25-30°C
Wash the wells
↓
Add 100 µL of 1x 14-3-3 Gamma Detection Antibody
↓ Incubate for 60 minutes at 25-30°C
Wash the wells
↓
Add 100 µL of 1x HRP conjugated Anti-IgG Antibody
↓ Incubate for 60 minutes at 25-30°C
Wash the wells
↓
Add 100 µL of Substrate Reagent
↓ Incubate for 10-20 minutes at 25-30°C
Add 100 µL of Stop Solution
↓
Measure absorbance at 450 nm

Materials Provided

All samples and standards should be assayed in duplicate. The following components are supplied and are sufficient for the one 96-well microplate kit.

Microplate: One microplate supplied ready to use, with 96 wells (12 strips of 8-wells) in a foil, zip-lock bag with a desiccant pack. Wells are coated with anti-14-3-3 Gamma antibody.

10X Wash Buffer: One bottle containing 100 mL of 10X wash buffer that contains Tween®-20

Sample/Standard Dilution Buffer: One bottle containing 50 mL of 1X Sample/Standard Dilution Buffer; use for the dilutions of sample and 14-3-3 Gamma Standard. Ready to use.

14-3-3 Gamma Standard: One vial containing X* AU of human 14-3-3 Gamma. Lyophilized.
***The amount is changed depending on lot. See the real "User's Manual" included in the kit box.**

100X 14-3-3 Gamma Detection Antibody: One vial containing 140 uL of anti-14-3-3 Gamma antibody.

Detection Antibody Dilution Buffer: One bottle containing 12 mL of Detection Antibody Dilution Buffer; use for the dilution of 100X 14-3-3 Gamma Detection Antibody. Ready to use.

100X HRP conjugated Anti-IgG Antibody: One vial containing 140 uL of HRP (horseradish peroxidase) conjugated anti-IgG antibody. Ready to use.

Conjugate Dilution Buffer: One bottle containing 12 mL of Conjugate Dilution Buffer; use for the dilution of 100X HRP conjugated Anti-IgG Antibody. Ready to use.

Substrate Reagent: One bottle containing 20 mL of the chromogenic substrate, tetra-methylbenzidine (TMB). Ready to use.

Stop Solution: One bottle containing 20 mL of 1 N H_2SO_4. Ready to use.

Materials Required but not Provided

- **Pipettors:** 2-20 µL, 20-200 µL and 200-1,000 µL precision pipettors with disposable tips.
- **Precision repeating pipettor**
- **Orbital microplate shaker**
- **Microcentrifuge and tubes** for sample preparation.
- **Vortex mixer**
- **(Optional) Microplate washer:** Manual washing is possible but not preferable.
- **Plate reader** capable of measuring absorbance in 96-well plates at dual wavelengths of 450 nm/540 nm. Dual wavelengths of 450/550 or 450/595 nm can also be used. The plate can also be read at a single wavelength of 450 nm, which will give a somewhat higher reading.
- **(Optional) Software package facilitating data generation and analysis**
- **500 or 1,000 mL graduated cylinder**.
- **Reagent reservoirs**
- **Deionized water of the highest quality**
- **Disposable paper towels**

Precautions and Recommendations

- **Although we suggest to conduct experiments as outlined below, the optimal experimental conditions will vary depending on the parameters being investigated, and must be determined by the individual user.**
- Allow all the components to come to room temperature before use.
- All microplate strips that are not immediately required should be returned to the zip-lock pouch, which must be carefully resealed to avoid moisture absorption.
- Do not use kit components beyond the indicated kit expiration date.
- Use only the microtiter wells provided with the kit.
- Rinse all detergent residue from glassware.
- Use deionized water of the highest quality.
- Do not mix reagents from different kits.
- The buffers and reagents in this kit may contain preservatives or other chemicals. Care should be taken to avoid direct contact with these reagents.
- Do not mouth pipette or ingest any of the reagents.
- Do not smoke, eat, or drink when performing the assay or in areas where samples or reagents are handled.
- Dispose of tetra-methylbenzidine (TMB) containing solutions in compliance with local regulations.
- Avoid contact with the acidic Stop Solution and Substrate Solution, which contains hydrogen peroxide.
- Wear gloves and eye protection when handling immunodiagnostic materials and samples of rat origin, and these reagents. In case of contact with the Stop Solution and the Substrate Solution, wash skin thoroughly with water and seek medical attention, when necessary.
- **Biological samples may be contaminated with infectious agents. Do not ingest, expose to open wounds or breathe aerosols. Wear protective gloves and dispose of biological samples properly.**
- **CAUTION: Sulfuric Acid is a strong acid. Wear disposable gloves and eye protection when handling Stop Solution.**

Sample Collection and Storage

CSF (Cerebrospinal fluid): Centrifuge CSF samples at 4°C for 15 minutes at 10,000 x g to remove insoluble impurities. Assay immediately or store the samples on ice for a few hours before assaying. Aliquots of the samples may also be stored at below -70°C for extended periods of time. Avoid repeated freeze-thaw cycles.

Cell lysate:

1. Harvest and pellet cells by centrifugation using standard methods.

2. Resuspend the cell pellet with an appropriate extraction buffer (for example; 20 mM HEPES-KOH, pH 7.5, 250 mM NaCl, 0.1 % NP-40, 2 mM $CaCl_2$, 1 mM EDTA, 0.2 mM PMSF, 1 µg/mL pepstatin, 0.5 µg/mL leupeptin, 0.5 mM DTT) and lyse the resuspended cells using either a Dounce Homogenizer, sonication, or three cycles of freezing and thawing.

3. Transfer extracts to microcentrifuge tubes and centrifuge at 15,000 rpm for 10 minutes at 4°C.

4. Aliquot cleared lysate to a clean microfuge tube.

5. Assay immediately or store the samples on ice for a few hours before assaying. Aliquots of the samples may also be stored at below -70°C for extended periods of time. Avoid repeated freeze-thaw cycles.

Other biological samples: MBL has not tested.
(*e.g.* Remove any particulates by centrifugation and assay immediately or aliquot and store samples at below -70°C. Avoid repeated freeze-thaw cycles. Individual users should determine appropriate conditions when using other types of samples.)

Detailed Protocol

The MBL Research Product **CircuLex 14-3-3 Gamma ELISA Kit** is provided with removable strips of wells so the assay can be carried out on separate occasions using only the number of strips required for the particular determination. Since experimental conditions may vary, an aliquot of the 14-3-3 Gamma Standard within the kit, should be included in each assay as a calibrator. Disposable pipette tips and reagent troughs should be used for all liquid transfers to avoid cross-contamination of reagents or samples.

Preparation of Working Solutions

All reagents need to be brought to room temperature prior to the assay. Assay reagents are supplied ready-to-use, with the exception of **10X Wash Buffer**, **100X 14-3-3 Gamma Detection Antibody**, and **14-3-3 Gamma Standard**.

1. Prepare a working solution of **1X Wash Buffer** by adding 100 mL of the **10X Wash Buffer** to 900 mL of deionized (distilled) water (ddH_2O). Mix well. Store at 4°C for two weeks or -20°C for long-term storage.

2. Prepare working solutions of **1X 14-3-3 Gamma Detection Antibody** by 100-fold diluting the **100X 14-3-3 Gamma Detection Antibody** with the **Detection Antibody Dilution Buffer**, *e.g.* 10 µL of 100X 14-3-3 Gamma Detection Antibody + 990 µL of Detection Antibody Dilution Buffer and mix well, and **1X HRP conjugated Anti-IgG Antibody** by 100-fold diluting the **100X HRP conjugated Anti-IgG Antibody** with the **Conjugate Dilution Buffer** in the same manner. Prepare the solutions just before using and discard unused one.

3. Reconstitute **14-3-3 Gamma Standard** with X* **mL** of **Sample/Standard Dilution Buffer**. The concentration of the 14-3-3 Gamma should be **64,000 AU/mL**, which is referred as the **Master Standard**. Make aliquots and store at below -70°C. Avoid repeated freeze-thaw cycles.
   ***The amount is changed depending on lot. See the real "User's Manual" included in the kit box.**

   Prepare Standard Solutions as follows:
   Use the **Master Standard** to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 16,000 AU/mL Standard Solution serves as the highest standard solution (Std.1). The **Dilution Buffer** serves as the zero standard solution (Blank).

Standard Solutions	Volume of Standard	Dilution Buffer	Final Concentration
Std.1	150 µL of Master Standard (64,000 AU/mL)	450 µL	16,000 AU/mL
Std.2	300 µL of Std. 1 (16,000 AU/mL)	300 µL	8,000 AU/mL
Std.3	300 µL of Std. 2 (8,000 AU/mL)	300 µL	4,000 AU/mL
Std.4	300 µL of Std. 3 (4,000 AU/mL)	300 µL	2,000 AU/mL
Std.5	300 µL of Std. 4 (2,000 AU/mL)	300 µL	1,000 AU/mL
Std.6	300 µL of Std. 5 (1,000 AU/mL)	300 µL	500 AU/mL
Std.7	300 µL of Std. 6 (500 AU/mL)	300 µL	250 AU/mL
Blank	-	300 µL	0 AU/mL

Note: Do not use a Repeating pipette. Change tips for every dilution. Wet tip with Dilution Buffer before dispensing. Unused portions of Master Standard should and Standard Solutions be aliquoted and stored at below -70°C immediately. Avoid multiple freeze and thaw cycles.

Sample Preparation

Dilute samples with **Sample/Standard Dilution Buffer**.
- CSF samples may be required 5- and 40-fold dilution.
- Cell lysates might be required 10- to 1,000-fold dilution, though it depends on each sample.

Assay Procedure

1. Remove the appropriate number of microtiter wells (strips) from the foil pouch and place them into the well holder. Return any unused wells to the foil pouch, refold, seal with tape and store at 4°C.

2. Dilute samples with **Sample/Standard Dilution Buffer**. (See "Sample Preparation" above.)

3. Pipette **100 µL** of **Standard Solutions (Std1-Std7, Blank)** and **diluted samples** in duplicates, into the appropriate wells.

4. Incubate the wells **at 25-30°C for 60 minutes**.

5. Wash 4-times by filling each well with **1X Wash Buffer** (350 µL) using a squirt bottle, multi-channel pipette, manifold dispenser or microplate washer.

6. Add **100 µL** of **1X 14-3-3 Gamma Detection Antibody** into each well.

7. Incubate the wells **at 25-30°C for 60 minutes**.

8. Wash 4-times by filling each well with **1X Wash Buffer** (350 µL) using a squirt bottle, multi-channel pipette, manifold dispenser or microplate washer.

9. Add **100 µL** of **1X HRP conjugated Anti-IgG Antibody** into each well.

10. Incubate the wells **at 25-30°C for 60 minutes**.

11. Wash 4-times by filling each well with **1X Wash Buffer** (350 µL) using a squirt bottle, multi-channel pipette, manifold dispenser or microplate washer.

12. Add **100 µL** of **Substrate Reagent** into each well. (Avoid exposing the well to direct sunlight. Covering the plate with *e.g.* aluminum foil is recommended.) Return Substrate Reagent to 4°C immediately after the necessary volume is removed.

13. Incubate the wells **at 25-30°C for 10-20 minutes**. This incubation time may be extended up to 30 minutes if the reaction temperature is relatively low.

14. Add **100 µL** of **Stop Solution** to each well in the same order as the previously added Substrate Reagent.

15. Measure absorbance in each well using a spectrophotometric microplate reader at dual wavelengths of 450/540 nm. Dual wavelengths of 450/550 or 450/595 nm can also be used. Read the microplate at 450 nm if only a single wavelength can be used. Wells must be read within 30 minutes of adding the Stop Solution.

Note-1: 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.

Note-2: Reliable standard curves are obtained when either O.D. value doesn't exceed 0.2 for the zero standard solution (Blank), or 2.8 for the highest standard solution (Std.1).

Note-3: If the microplate reader is not capable of reading absorbance greater than the absorbance of the highest standard, perform a second reading at 405 nm. A new standard curve, constructed using the values measured at 405 nm, is used to determine 14-3-3 Gamma concentration of off-scale samples. The readings at 405 nm should not replace the on-scale readings at 450 nm.

Calculations

Average the duplicate readings for each standard, control and sample, and subtract the optical density of the average zero standard. Plot the optical density versus the concentration of standards and draw the best curve. Most microtiter plate readers perform automatic calculations of analyte concentration. The standard curve fits best to a sigmoidal four-parameter logistic equation. The results of unknown samples can be calculated with any computer program having a four-parameter logistic function.

A standard curve is also to be constructed by plotting the absorbance (Y) versus log of the known concentration (X) of standards, using a cubic function. Alternatively, the logit log function can be used to linearize the standard curve (i.e. logit of optical density (Y) is plotted versus log of the known concentration (X) of standards). To determine the concentration of each sample, first find the optical density on the y-axis and extend a horizontal line to the standard curve. At the point of intersection, extend a vertical line to the x-axis and read the corresponding concentration.

If the samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

Measurement Range

The measurement range is 250 AU/mL to 16,000 AU/mL. Any sample reading higher than the highest standard should be diluted with Dilution Buffer in higher dilution and re-assayed. Dilution factors need to be taken into consideration in calculating the human 14-3-3 Gamma concentration.

Troubleshooting

1. All samples and standards should be assayed in duplicate, using the protocol described in the **Detailed Protocol**. Incubation times or temperatures significantly different from those specified may give erroneous results.

2. Poor duplicates, accompanied by elevated values for wells containing no sample, indicate insufficient washing. If all instructions in the **Detailed Protocol** were followed accurately, such results indicate a need for washer maintenance.

3. Overall low signal may indicate that desiccation of the wells has occurred between the final wash and addition of Substrate Reagent. Do not allow the wells to dry out. Add Substrate Reagent immediately after wash.

Reagent Stability

All of the reagents included in the MBL Research Product **CircuLex 14-3-3 Gamma ELISA Kit** have been tested for stability. Reagents should not be used beyond the stated expiration date. Upon receipt, kit reagents should be stored at 4°C, except the reconstituted 14-3-3 Gamma Standard (Master Standard) must be stored at below -70°C. The Microplate should be stored in the original foil bag sealed by the zip lock and containing a desiccant pack.

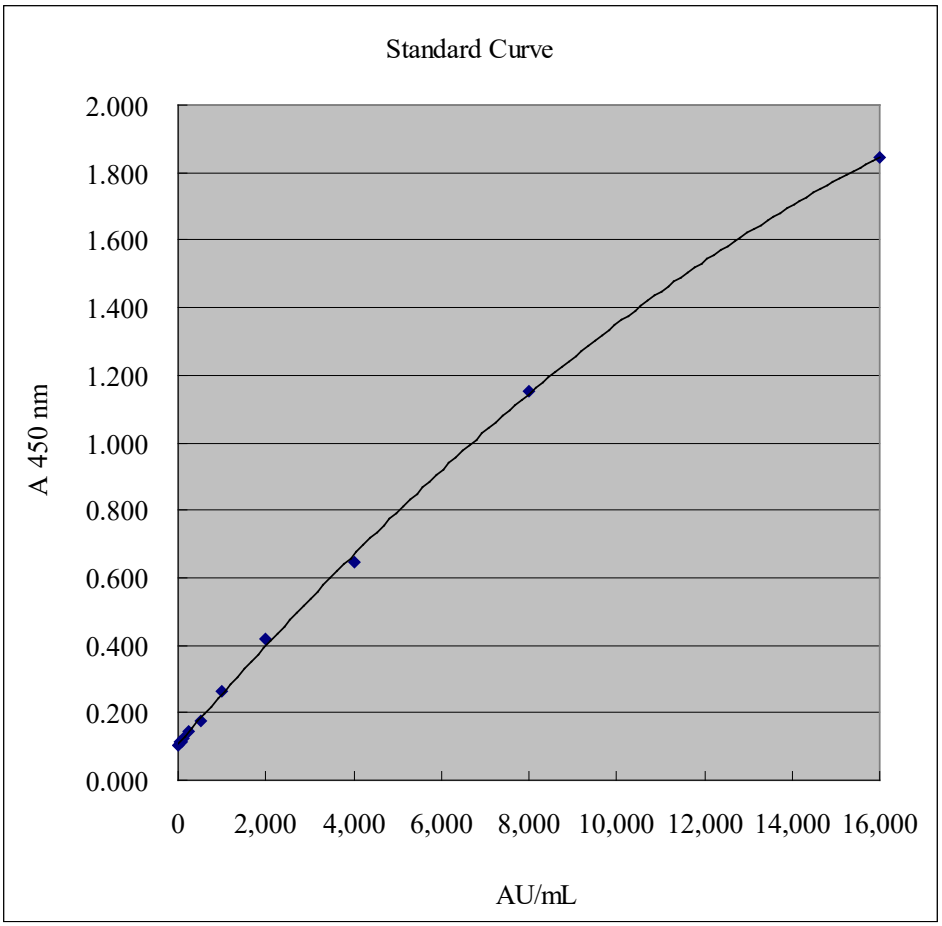
Assay Characteristics

1. Sensitivity

Five assays were evaluated and the minimum detectable dose (MDD) of 14-3-3 Gamma.
The MDD (defined as such a concentration of 14-3-3 Gamma giving absorbance higher than mean absorbance of blank* plus three standard deviations of the absorbance of blank: A blank + 3SD blank) is better than 250 AU/mL of sample.

* Standard/Sample Dilution Buffer was pipetted into blank wells.

Typical standard curve



2. Precision

Intra-assay Precision (Precision within an assay)

Three samples of Raji (human Burkitt's lymphoma cell line) cell lysate known concentration were tested twelve times on one plate to assess intra-assay precision.

• Intra-assay (Within-Run, n=12); CV= 1.04 - 4.35 %

14-3-3 Gamma conc. (AU/mL)

	Low	Middle	High
1	2,271.90	4,298.20	9,317.24
2	2,193.38	4,210.38	8,882.98
3	2,201.48	4,192.23	8,879.64
4	2,228.84	4,164.24	8,585.26
5	2,222.21	4,151.33	8,610.61
6	2,236.05	4,233.14	8,680.03
7	2,243.23	4,650.08	8,691.03
8	2,210.10	4,579.00	8,606.62
9	2,227.44	4,553.39	9,401.07
10	2,244.41	4,634.24	9,274.35
11	2,260.28	4,398.29	8,624.60
12	2,244.70	4,404.98	8,677.69
MEAN	2,232.00	4,372.46	8,852.59
S.D.	23.12	190.37	305.29
CV%	1.04%	4.35%	3.45%

Inter-assay Precision (Precision between assays)

Three samples of Raji (human Burkitt's lymphoma cell line) cell lysate known concentration were tested in five separate assays to assess inter-assay precision.

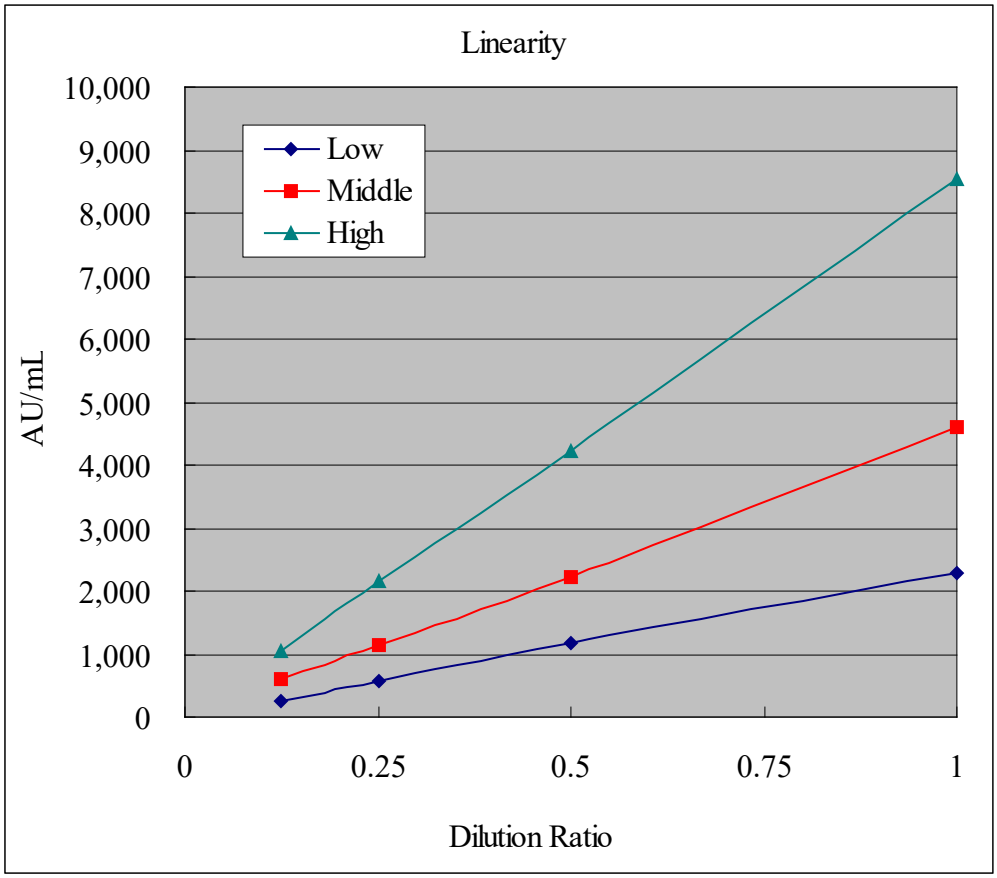
• Inter-assay (Run-to-Run, n=5); CV= 3.85 - 6.05 %

14-3-3 Gamma conc. (AU/mL)

	Low	Middle	High
1	2,269.34	4,255.06	8,571.22
2	2,312.22	4,371.21	9,430.12
3	2,333.07	4,666.39	9,755.37
4	2,553.49	4,563.30	9,432.60
5	2,290.10	4,608.39	8,546.55
MEAN	2,351.64	4,492.87	9,147.17
S.D.	115.33	173.04	553.15
CV%	4.90%	3.85%	6.05%

3. Linearity

To assess the linearity of the assay, Raji (human Burkitt's lymphoma cell line) cell lysate samples containing 14-3-3 Gamma were serially diluted with the Standard/Sample Dilution Buffer to produce samples with values within the dynamic range of the assay.



Example of Test Results

Fig.1 Measurement of concentrations of 14-3-3 Gamma in CSF of patients with CJD*

Units of 14-3-3 Gamma concentration in CSF from patients with CJD and non-CJD neurological disorders were measured by the CircuLex 14-3-3 Gamma ELISA Kit.

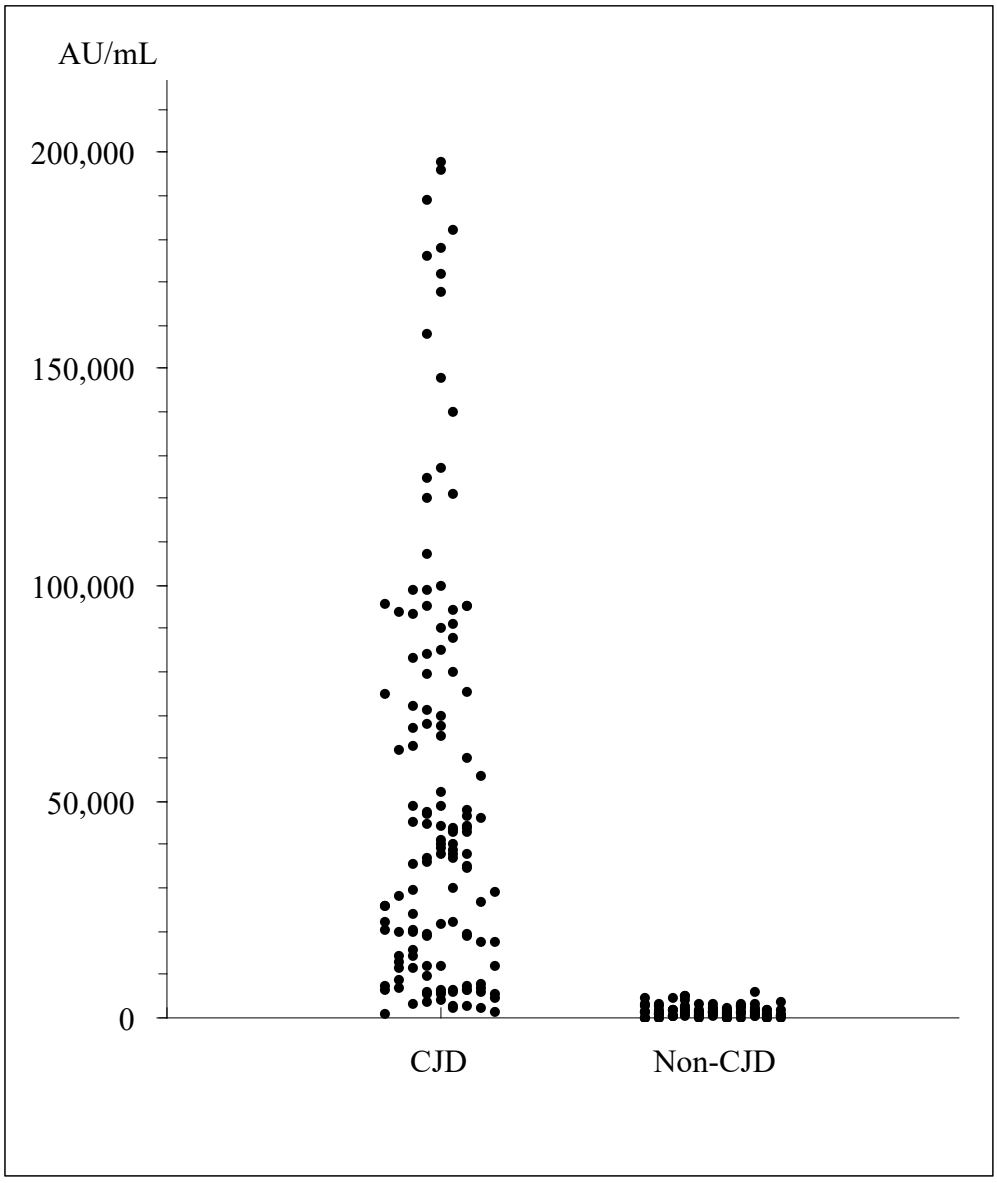
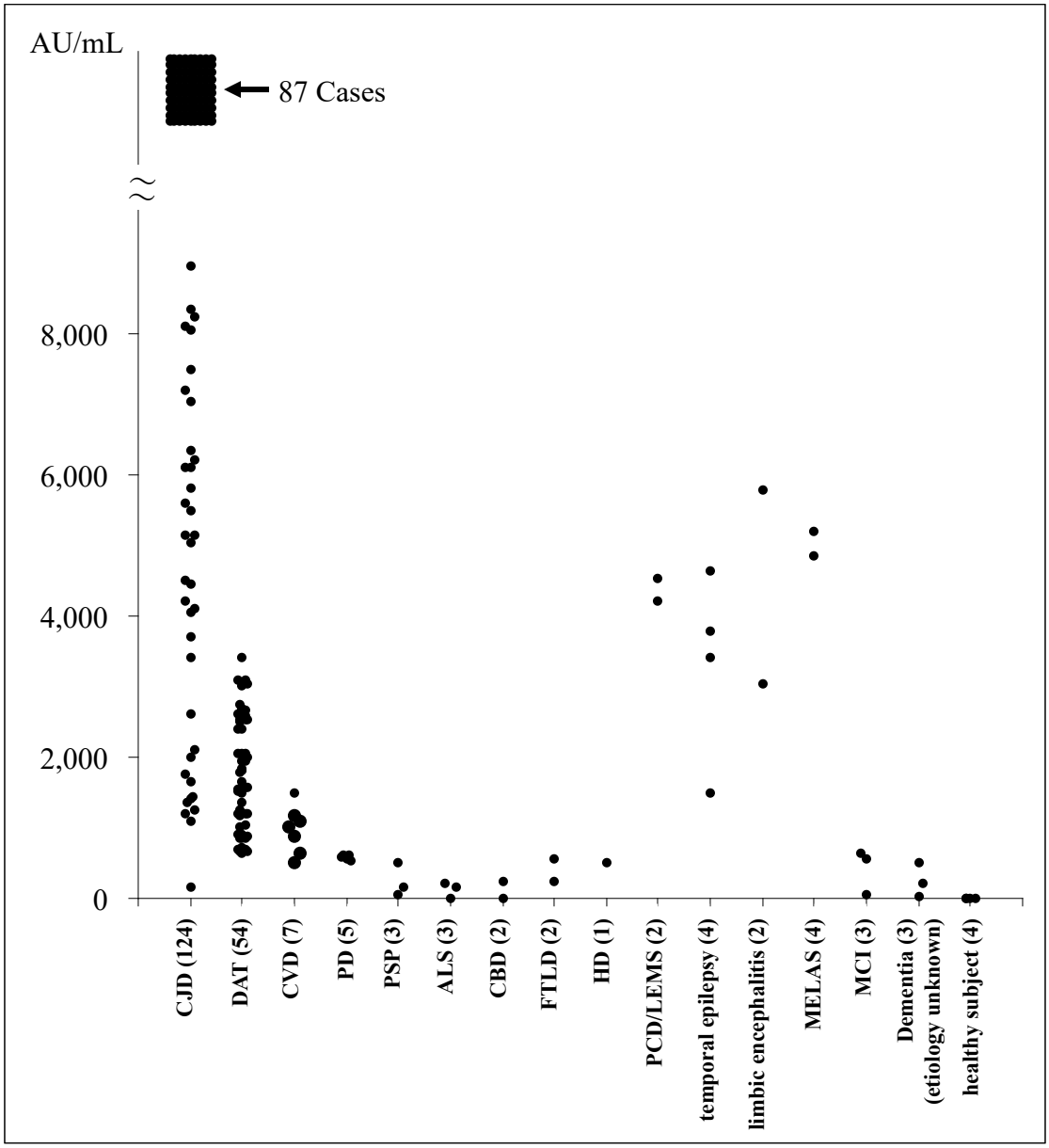


Fig.2 Detailed analysis of 14-3-3 Gamma level in CSF of CJD and other neurological disorders patients in the range of lower concentration*

14-3-3 Gamma levels in CSF from CJD patients (n=124) and other neurological disorders patients (n=99) were measured by the CircuLex 14-3-3 Gamma ELISA Kit and the result was shown at lower concentration.



(The numbers of each disorder are shown in parentheses.)

Abbreviations of disorders
CJD: Creutzfeldt-Jakob disease
DAT: Dementia of Alzheimer's type

CVD: Cerebral Vascular Disorder
PD: Parkinson's disease
PSP: progressive supranuclear palsy
FTLD: frontotemporal lobular degeneration
HD: Huntington's disease
CBD: corticobasal degeneration
ALS: amyotrophic lateral sclerosis
MELAS: Mitochondrial myopathy, Encephalopathy, Lactic Acidosis, Stroke-like episodes
PCD: paraneoplastic cerebellar degeneration
LEMS: Lambert-Eaton myasthenic syndrome
MCI: mild cognitive impairment

*** The data in Fig.1 and Fig.2 were kindly provided by Dr. Yuki Matsui[1] and Dr. Katsuya Satoh[2].**
([1]Department of Pharmaceutical Care and Health Sciences, Faculty of Pharmaceutical Sciences, Fukuoka University, [2]Department of Molecular Microbiology and Immunology,Graduate School of Biomedical Science, Nagasaki University. See the reference (8) for details.)

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For more information, please visit our web site**.**
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E-mail: support@mbl.co.jp