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Questionable accuracy of four ELISA kits in serum Netrin-1 measurement

<https://doi.org/10.1515/labmed-2024-0028>

Received February 12, 2024; accepted May 16, 2024;

published online June 10, 2024

Abstract

Objectives: Altered serum Netrin-1 levels have been widely reported in cancer and other clinical diseases and they are often measured by commercial ELISA kits. However, we found the questionable results using these kits and therefore performed this simple study to evaluate their accuracy in detection of serum Netrin-1.

Methods: Four commonly used commercial kits were collected. The kit standards were serially diluted or spiked into serum samples. The cells with confirmed expression of Netrin-1 and their culture medium, as well as the Netrin-1 controls of each kit were used for the kits to detect. The cell lysate samples and the kit controls were also blotted on a nitrocellulose membrane for detection antibodies of each kit to probe.

Results: Detection of the Netrin-1 standards in serum by each kit were all affected. Only one kit was able to detect Netrin-1 in the cell lysate or medium. No ELISA kits could detect all Netrin-1 controls of the four kits. None of the detection antibodies correctly probed Netrin-1 in the dot blot.

Conclusions: The accuracy of these four Netrin-1 ELISA kits is under question. Reported serum Netrin-1 levels based on measurements by these kits need be carefully interpreted.

Keywords: Netrin-1; ELISA; accuracy; serum interference

Introduction

Netrin-1 is initially discovered as a growth cue that regulates axon guidance. It is a secreted protein that consists 604 amino acids and is expressed at relatively low levels in adult human tissues in adults [1]. Besides its role in axon guidance, Netrin-1 has been found to be involved in tumorigenesis, inflammation, neurodegeneration and other clinical disorders [2–4].

Altered Netrin-1 levels in serum, urine or types of body fluids are associated with a large number of these clinical diseases, atherosclerosis [5, 6], obesity and diabetes [7–9], kidney injury [10, 11], brain damage and hemorrhage [12–14], periodontitis [15, 16], acute coronary syndrome [17], sclerosis [18], preeclampsia [19–22], ischemic stroke and its complications [23–26], post-stroke depression [27], and delayed neurological sequelae in unintentional carbon monoxide poisoning [28], etc. Increased Netrin-1 is also found in blood in cancer, including breast, renal, prostate, liver, meningioma, pituitary adenoma, and glioblastoma cancers than it in controls [29]. It is also increased in gastric and lung cancers and becomes reduced after chemotherapy [30, 31]. Recent studies propose serum Netrin-1 as a novel biomarker in both lung and colorectal cancers [32–34].

We have recently found that the Netrin-1 protein level is significantly increased in the brain tissue of patients with Alzheimer's disease (AD), and highly correlated with A β in their levels, which is also seen in the AD mouse model [35]. This prompted us to measure Netrin-1 in the serum of both patients and the mouse models. However, when we used one of the commercial ELISA kits according to the reports above, we found the results questionable and contradictory. Therefore, we performed this simple study to evaluate the accuracy of the commonly used Netrin-1 ELISA kits in reported studies for better understanding of Netrin-1 as a serum biomarker in clinical diseases.

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Materials and methods

All research protocols were approved by the Ethics Committee of Nanjing Drum Tower Hospital, and all experiments were in accordance with relevant guidelines and regulations. Informed consents were obtained from all subjects or their legal guardians.

The sandwich ELISA kits and the assay procedures

Four Netrin-1 ELISA kits were recruited, including Kit 1 (Thermo Fisher Scientific, EM56RB), Kit 2 (Cusabio, CSB-E11899h), Kit 3 (Cloud-Clone Corp, SEB827Hu), and Kit 4 (LSBio, LS-F5003). The standard stock in Kit 1 is 20,000 pg/mL and the standards in other three kits are all 2,000 pg/mL. The detection antibodies of the four kits were all biotinylated. All assay procedures followed the instructions of each kit. All ELISA measurements in this study were made in the same batch on a plate with the serial dilutions of the standard in the diluent as the reference curve for quantitation.

Clinical serum sample collection

The serum sample used for dilution of the kit standards were from the mixture of 10 individual serum specimens from individuals after they had finished routine health examination and had no abnormal results in clinical laboratory tests including infection screening. These individuals included six males and four females with ages ranging from 24 to 45 years old and all have normal body mass indexes (18.5–24.9). The other 16 individual serum samples were patients with different types of cancers as reported to have altered serum Netrin-1 levels after screening by Kit 1 to include samples with high, moderate, and low results respectively. All serum samples were collected within 6 h after the blood was drawn, and were centrifuged at 3,000 rpm for 10 min for serum collection and stored at -80°C until use. The clinical serum sample collection was approved by our institutional research ethics board.

Serum interference

The Netrin-1 standard of each kit was serially diluted in the mixed serum sample, and also similarly diluted in the diluent of the same kit as the control. For example, in Kit 1, its Netrin-1 standard (20,000 ng/mL) was diluted to 8,000, 3,200, 1,280, 512, 205, 82 pg/mL in the diluent and was also diluted to

the same concentrations in the mixed serum. Comparison of the difference between the results of standard in the diluent and it in the serum for interference analysis.

Cell culture and Netrin-1 expression

HEK293T cell were cultured in the DMEM medium containing 10 % fetal bovine serum in the presence of antibiotics at 37°C with 5 % CO_2 . The DNA sequence that encodes for Netrin-1 (O95631/Uniprot, 604 amino acids) with a Kozac sequence (GCCACC) at the 5'-terminus to enhance translation and a DDK tag (DYKDDDDK) at the C-terminus for protein identification and purification, was constructed into the vector pcDNA3.1(+). Transfection was done with the reagent ExFect (Vazyme). Cells and the medium were collected after 24 h. Cells were lysed in RIPA buffer in the presence of protease inhibitor cocktail (Roche) and stored at -80°C . The cell lysates were diluted by 10-fold in the TBST (Tris-buffered saline with 0.05 % Tween-20) prior to detection by ELISA in case the strong detergents in RIPA might possibly detach the coated antibodies from the ELISA plate.

Western blot

Protein concentrations in the cell lysate samples were determined by the BCA method (Pierce) and then 20 μg proteins of each sample were loaded for SDS-PAGE (4–12 % gel, GenScript). Proteins were then electro-transferred onto a nitrocellulose membrane and then stained with Ponceau S before being blocked with 5 % non-fat milk. The antibody used to blot Netrin-1 was from Abcam (ab126729) and diluted by 1:1,000 in 3 % BSA.

Recovery analysis

To each of the first eight clinical serum samples, add the Netrin-1 standard of each kit (vol/vol=9/1) to 2,000 pg/mL (Kit 1) or 200 pg/mL (the other three kits). The original serum samples and the spike-in serum samples were measured altogether. Recovery (%)=(the measured result – the result of the original serum)/the concentration of spiked-in standard (2,000 or 200 pg/mL).

Dot immunoblot

1 μL of each protein sample was added onto the nitrocellulose membrane and let it dry in air for 5–10 min. The

membrane was soaked in 5 % formic acid and stained by Ponceau S, and then washed for blocking with 5 % non-fat milk. The membrane was then incubated with the Netrin-1 antibody (1:1,000 dilution in 3 % BSA) or the ELISA kit detection antibodies (diluted as the kit instructions suggest).

Statistical analysis

The data correlation, regression, and recovery analyses were carried out in the Microsoft Excel.

Results

Selection of ELISA kits

The information of the four ELISA kits is summarized in the Table 1. We initially purchased the kit 2 according to reports and used the human Netrin-1-expressing cells and the culture medium as the control samples, and serially diluted the kit standard in serum for testing of the interference to verify the kit accuracy. However, this kit failed both experiments. We then bought the kits 3

and 4 according to other reported studies, and again, these two kits also did not pass the two verification tests either.

Some important information about these kits (such as the source of the coated and detection antibodies, immunogen sequences against which these antibodies were raised, monoclonal or polyclonal, the antibody purification approaches, etc.) were not provided by these kits or the manufacture websites. The kit 2 was initially bought according to the extensive reported studies, but did not differentiate the cell samples that express or do not express Netrin-1 used as control samples, and it did not pass the serum interference test either. We then bought the kit 3 and 4, both of which displayed the same problems. We therefore turned to the reputable Thermo but they only sell mouse Netrin-1 kit. We aligned the mouse and human Netrin-1 protein amino acid sequences and they both are highly identical, only bearing five different amino acids spread widely which unlikely generating a significant impact on the antigenicity. Moreover, in the preliminary experiments, it could detect the human Netrin-1 expressed by the cultured cells. We thus included it as Kit 1 in this study for comparison together. All the four kits were analyzed together in this study.

Table 1: Information about the four commercial kits.

	Kit 1	Kit 2	Kit 3	Kit 4
Provider	Thermo Fisher Scientific	Cusabio	Cloud-Clone Corp	LSBio
Catalog number	EM56RB	CSB-E11899h	SEB827Hu	LS-F5003
Host species of coating and detection antibodies	Both from mouse	Not available	Not available	Not available
Immunogen sequence of coating and detection antibodies	Not available	Not available	Not available	Not available
Monoclonal/polyclonal type of coating and detection antibodies	Not available	Not available	Not available	Not available
Biotinylation of detection antibody	Yes	Yes	Yes	Yes
Detection range	90–20,000 pg/mL	31.25–2,000 pg/mL	31.2–2,000 pg/mL	31.2–2,000 pg/mL
Netrin-1 standard	20,000 pg/mL	2,000 pg/mL	2,000 pg/mL	2,000 pg/mL
Positive and negative controls	Not provided	Not provided	Not provided	Not provided
Sample type	Cell culture supernatants, plasma and serum	Cell culture supernatants, plasma, and serum	Cell culture supernates, cell lysates, plasma, serum, tissue homogenates, and other biological fluids	Cell culture supernates, cell lysates, plasma, serum, tissue homogenates, and other biological fluids
Reports that used this kit (PMID)	36400204	34897588; 36852451; 36812256; 35806983; 25903786; 28856010; 35806983; 37561046; 32474463; 35063900; 32912541; 32917498; 33546947; 30852966; 27067437	29156815; 32267322; 32474463; 37259741; 25154466	33891683; 36546321

Interference of serum on detection of Netrin-1 standards by each kit

All Netrin-1 standards serially diluted in the diluent of each own kit showed gradually decreasing chemiluminescent signals as expected (Figure 1A), and yielded reasonable linear relationships between the absorbances and the concentrations (Figure 1B). However, when the standards were diluted in the serum, the gradual reduction in signals and the linear relationships almost disappeared for the ELISA kit 1 and 2. This is probably because the serum as the diluent also generated signal and contributed to the overall absorbance. In comparison, in the Kit 3 and 4, the signals of the serially diluted standards in serum were clearly inhibited, indicating a suppressing effect of the serum on detection of these samples (Figure 1A and B). It is notable that the kits 1 and 2 could detect signals from the diluent serum while the other two kits (3 and 4) could not. It is notable that none of these kits could provide results of standards diluted in the

serum as expected (Figure 1C). The inconsistent results raise question about the detection accuracy of these kits.

Detection of expressed Netrin-1 in cells and culture medium

Netrin-1 was successfully expressed in HEK293T cells at relatively high level as compared to the control cells without being transfected (Figure 2A). This also suggested the antibody is specific to blot the Netrin-1 protein. When the lysate and the culture medium samples were detected by the four Netrin-1 ELISA kits, Kit 1 could detect the positive cell lysate and medium samples and well differentiate them from the negative controls (Figure 2B). However, the other three kits could not detect the Netrin-1-containing cell lysate or medium samples, or differentiate them from the negative controls, implicating questionable capability of detecting Netrin-1.

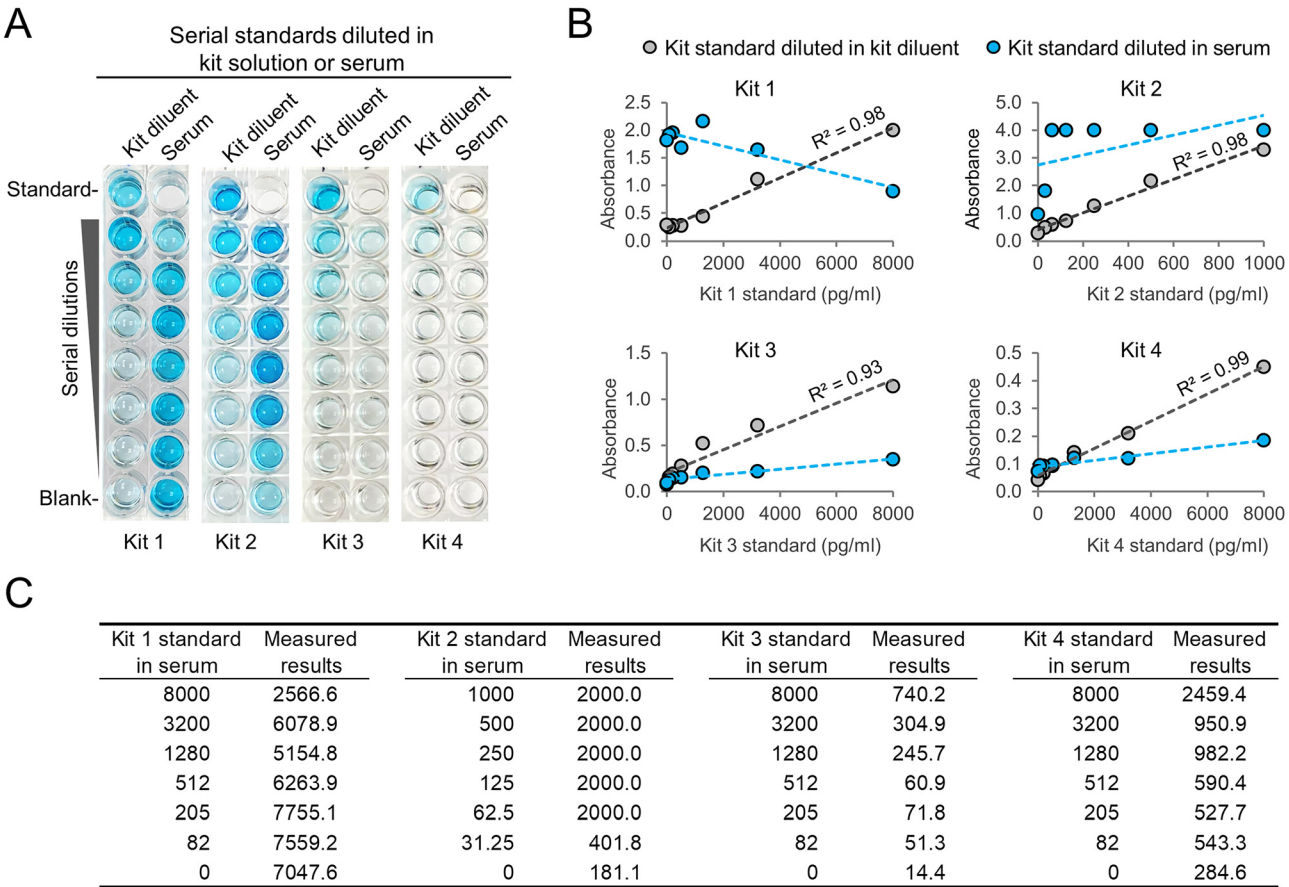


Figure 1: Serum interference in the four commercial Netrin-1 ELISA kits. (A) Serial dilutions of the Netrin-1 standard of each kit in the diluent of the kit or in the clinical serum. The serum is from the mixture of 10 clinically healthy individuals. For the standards diluted in the serum, the first well is empty because the first diluted sample is added to the second well to parallel wells on the left with the same dilutions of the standard. (B) Linear relationships of the measured ELISA results for comparison of the kit standards diluted in the kit diluent and those in the serum. (C) Quantitated results of each diluted standard in the serum. The results of standards diluted in the kit diluent were used as the calibration curve.

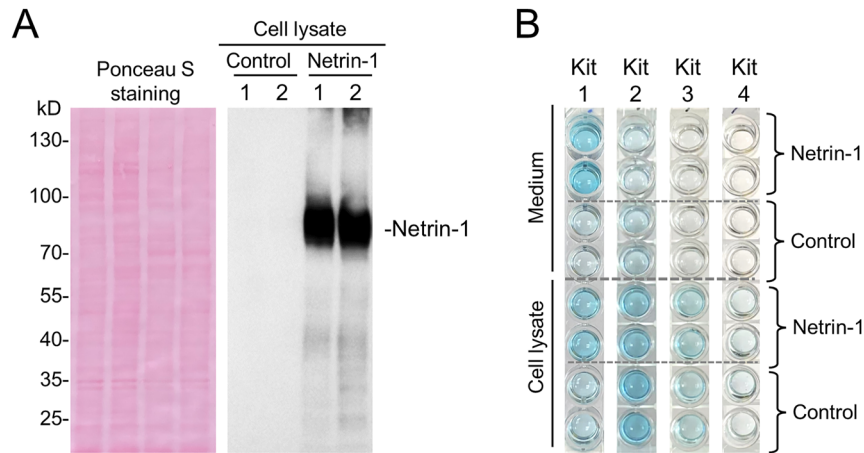


Figure 2: Detection of Netrin-1 expressed in cells. (A) Western blot of Netrin-1 expressed in HEK293T cells. The untransfected cells are used as controls. “1” and “2” are duplicated samples in transfection. (B) ELISA measurements of Netrin-1 in the medium or the lysate samples from cells that express Netrin-1.

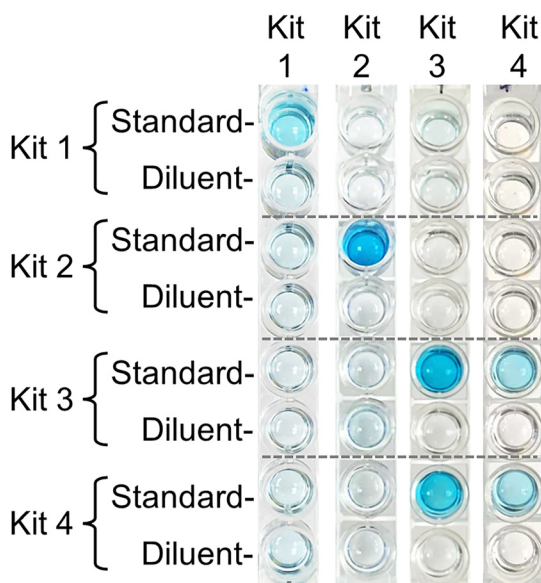


Figure 3: Detection of control samples of all four commercial kits by each ELISA assay. The undiluted standard and the diluent of each kit are added.

Detection of all kit Netrin-1 controls

When the positive controls and the negative controls of all these four kits were detected by each of these ELISA kits, none of these kits could recognize all the Netrin-1 controls. Kit 1 and Kit 2 only recognized its own positive controls, and Kit 3 and Kit 4 could detect both Netrin-1 controls of them but not the positive controls from Kit 1 and Kit 2 (Figure 3).

Detection of clinical serum samples and recovery analysis

Sixteen clinical serum samples were collected and detected by each of these four kits. Only the results of the kit 3 and kit

4 were seemed consistent but the signals of Kit 4 were weaker (Figure 4A). In contrast, ELISA signals of kit 1 and kit 2 were not comparable at all, and not consistent with the signals of kits 3 and 4 either. The regression analyses showed only the results between the kits 3 and 4 were significantly correlated but not between any two of other kits (Figure 4B). In detection of the eight samples with the spiked-in kit standard, kits 1 and 2 did not show increased ELISA signals as expected. Increased chemiluminescent signals could be observed in kits 3 and 4, but not consistent for all samples (Figure 4A). Although kits 3 and 4 showed better recovery rate than the other two kits, but none of these kits demonstrated an averaged recovery rate within the range of 10 % away to 100.0 % (90–110 %) (Figure 4C).

Detection of all kit Netrin-1 antibodies

In dot blots of their own Netrin-1 controls of each kit, the sample of Netrin-1 expressed in HEK293T cells could be successfully probed by an antibody that targets Netrin-1 specifically (Figure 2A). However, none of the Netrin-1 controls of the four kits could be detected by this antibody, suggesting low levels of the Netrin-1 protein in these controls or they were distinct and could not be recognized at all. Besides, none of the four Netrin-1 detection antibodies of each kit could well differentiate the cell samples that expressed Netrin-1 or not, nor could they detect the negative and positive controls of all four kits correctly (Figure 5).

Discussion

In this study, we simply analyzed the accuracy of the four commercial Netrin-1 ELISA kits commonly used in research. We found that serum had significant interference on all

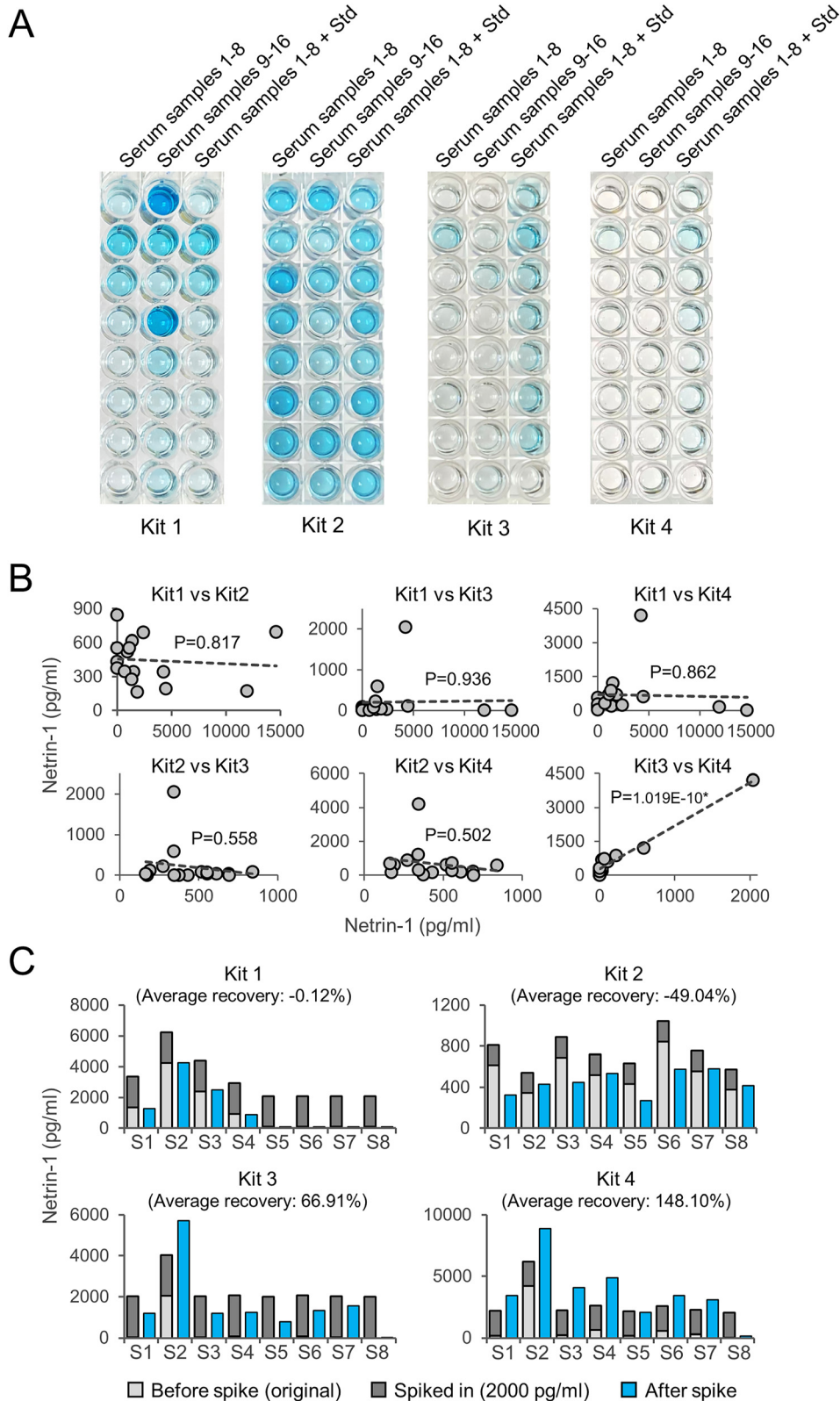


Figure 4: Detection of individual clinical serum samples and the recovery analysis. (A) ELISA assays of 16 clinical serum samples by each of the four commercial kits. The standard of each kit is spiked by 1/9 vol into the first eight serum samples. The final concentration of the standard in each serum sample is 2,000 pg/mL for Kit 1, and is 200 pg/mL for the other three kits. (B) Correlation of the ELISA results of the 16 clinical serum samples by these four kits. (C) The results of the first eight clinical samples before and after spike-in of the Netrin-1 standard of each kit. S1–S8: eight clinical serum samples.

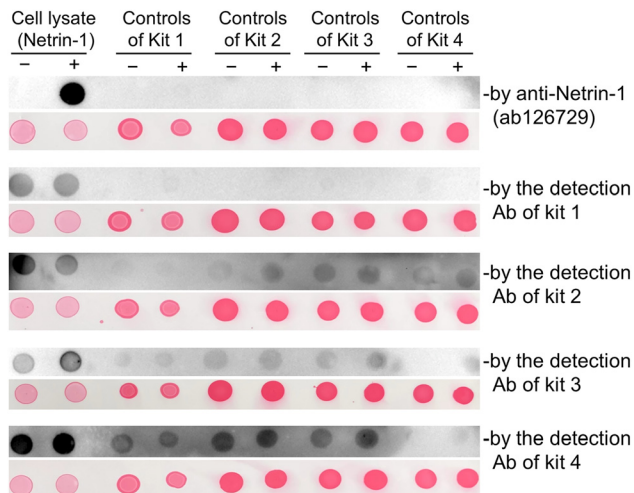


Figure 5: Dot blot of Netrin-1 expressed in cultured cells and the control samples of each kit by anti-Netrin-1 and the detection antibody of kit. Cell lysate samples are from HEK293T with or without expression of Netrin-1. The membrane are stained by Ponceau S after coating of protein samples.

these kits, only one kit can detect Netrin-1 expressed in cultured cells, and results of these kits were largely inconsistent. These suggest that the accuracy of these kits in detection of serum Netrin-1 is questionable.

It is notable that the results of kits 3 and 4 are very consistent and thus are likely from a same manufacture. According to the information from the website of the kit 3, CLOUD-CLONE CORP might be the original design manufacturer and the kit might be branded by LSBio. However, this is not indicated in the instructions or the website of kit 4. Lack of source information is a general issue in commercial kits for research use.

Although the kit 1 suggests that it can be used to measure serum Netrin-1, it does not provide any data on the kit booklet of their performance on serum samples. Like most commercial ELISA kits, the kit 1 only provides a curve of the Netrin-1 standard serially diluted in diluent. Although the diluent might include albumin as the common carrier, it is still largely different from the serum in both protein concentration and complexity. Although information from the website indicates that the kit 2 has a recovery rate of about 90 % on serum samples, the results in our study does not support this (Figure 4A–C). In contrast, the kits 3 and 4 has better recovery rates on serum samples but they are still not close to about 100 % as indicated by the kit manuals. What is puzzling is that none of these kits can recognize the Netrin-1 standards from other kits except the kits 3 and 4 which seem to be from the same manufacture, suggesting that there is certainly an issue with the standards or the kits.

It is also notable that none of the reports had tested the accuracy of these kits before they were used to measure Netrin-1. There were no indications of any positive and negative control samples used when the samples of interests were measured. In consideration of the scientific rigor, the calibration curve using standards diluted in the diluent instead of serum is not suitable for quantification of the serum samples. Lack of validation or verification of commercial kits prior to use is a common problem in reported studies.

Establishing reliable ELISA immunoassay kits for clinical use is challenging. Besides the common factors that affect the quality of ELISA methods [36], other conditions also need be considered. Blasting the protein sequence of Netrin-1 in Pubmed and Uniprot (<https://www.uniprot.org/blast>) yielded about 70 proteins with the same domains or highly similar sequences, including netrins (e.g., Netrin-2 to -5, G1 and G2) and the family of other laminin-like secreted proteins that Netrin-1 belongs to. If both the capturing antibody and the detection antibody of the kit can recognize the shared sequences, then these proteins will also be detected, generating false-positive signals. If either of them binds the shared sequences, then these proteins will interfere with the specific binding of the antibody to Netrin-1, yielding false negative results. In reality, even a protein has no shared sequences with the target protein, it could also generate epitopes for antibodies to bind as they can be conformational and are not always sequence-specific.

Highly abundant proteins in serum could also be an important interfering factor to ELISAs. Antibodies binding to their target antigens do not follow the on-and off mode. The binding is actually concentration and affinity dependent. This means that an antibody can also bind to nonspecific antigens and the binding affinity is just much weaker. If a nontarget protein can be bound to an antibody even at ten-million-fold lower affinity, but the concentration of this proteins is at about mg/ml level in serum (e.g., albumin, immunoglobins, complements, etc.) and the target protein is only at ng/ml or even lower (like cytokines, growth factors, etc.), then this protein will interfere detection of this specific target significantly. This is probably the most common reason that detection of specific proteins is often suppressed in serum.

There are many other factors that could affect the reliability of ELISA kits. Therefore, any immunoassay kits need be validated and even certified prior to use, especially when they detect serum or plasma samples. By simple experiments as performed in this study, caveats of these ELISA kits can be easily found.

Conclusions

We have found the four commercial Netrin-1 ELISA kits often used in research have an accuracy issue in detecting the serum level of Netrin-1. Therefore, reported findings about serum levels of Netrin-1 based on these kits need be carefully reexamined. Validation of the assay kits is expected prior to use for clinical samples, especially sera.

Research ethics: All research protocols were approved by the Ethics Committee of Nanjing Drum Tower Hospital, and all experiments were in accordance with relevant guidelines and regulations.

Informed consent: Not applicable.

Author contributions: The authors have accepted responsibility for the entire content of this manuscript and approved its submission.

Competing interests: The authors state no conflict of interest.

Research funding: This work was partially supported by the National Natural Science Foundation of China (82172354, to BB), the Research Foundation of Jiangsu Provincial Commission of Health and Family Planning (M2021012, to BB), the Nanjing Medical Science and Technology Development Foundation (ZKX22013, to BB), the fundings for Novel technology and Clinical Trials from the Affiliated Drum Tower Hospital, Medical School of Nanjing University (XJSFZLX202331 and 2022-LCYJ-MS-02, to BB).

Data availability: The raw data can be obtained on request from the corresponding author.

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