RESEARCH ARTICLE

REVISED A highly sensitive enzyme-linked immunosorbent assay allows accurate measurements of brain-derived neurotrophic factor levels in human saliva

[version 2; peer review: 2 approved, 1 not approved]

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Abstract

Background

Hitherto, BDNF levels in humans have been primarily measured in serum and/or plasma where these levels are readily measurable, but primarily reflect the content of BDNF in blood platelets. By contrast, previous attempts to measure BDNF levels in readily accessible human body fluids such as saliva have been complicated by a lack of sensitivity and/or specificity of BDNF ELISAs (see Discussion). Recently, the suitability of a highly sensitive BDNF ELISA assay was validated using mouse plasma and serum where conventional BDNF ELISA fail to detect BDNF. In this report, we demonstrate that BDNF levels in human saliva are extremely low, in the low pg/mL range, yet detectable in all saliva samples tested.

Methods

Saliva samples were collected from healthy volunteers by a passive drool method. All samples were aliquoted and immediately frozen to keep at -80°C until use. At the time of use, the samples were thawed, centrifuged to remove any remaining particles and BDNF measurement conducted by using a previously validated BDNF ELISA assay (see below). Recombinant mature BDNF was used as a reference.

Results

Open Peer Review

Approval Status ✗ ✓ ✓

	1	2	3
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The intra-assay variability was in the range of CV = 1.8 to 4.9%. Saliva samples could be kept frozen at -80°C for 2 months until use for measurements, but more than 4 freeze and thaw cycles caused BDNF losses presumably due to structural change of the antigen. The measurements were not affected by the method of collection provided the samples were diluted at least 2-fold.

Conclusions

The results indicate that human saliva samples collected in a non-invasive fashion can be used as a source of material to try and correlate BDNF levels with human conditions of interest. These results also confirm those of an independent study published recently using the same BDNF ELISA kit to measure BDNF levels in human saliva samples.

Keywords
ELISA, BDNF, Saliva, Immunoassay, Analytical Method

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REVISED **Amendments from Version 1**

The central point of our manuscript is the use of a highly sensitive BDNF ELISA kit that has been validated in a previous study (PMID: 37173369) in which Want and colleagues documented both the specificity and sensitivity of this ELISA kit. This previous study demonstrated that the kit allows reliable measurements to be made of the very low levels of mature BDNF (mBDNF) in mouse serum or plasma. Importantly and unlike is the case in human samples for example, the values of mBDNF in mouse serum or plasma were found to be indistinguishable. This same ELISA allows mBDNF measurements in human saliva, while previously used BDNF ELISA kits reported typically did not have the necessary sensitivity and/or reported implausibly high BDNF levels in human saliva, suggesting a lack of specificity.

Based on peer reviewers' suggestions, we made the following changes:

- We replaced reference 2 with the most recent review article.
- We removed Nakagawa et al. and Kikuchi et al. from References since they did not discuss human saliva BDNF measurement.
- We added the details of sample collection time and volume for clarification.
- We included Tahiroglu et al. which discusses human saliva BDNF measurement and was published after our original submission of this abstract as the reference.
- We added the sentence "and significant number (up to 35%) of saliva samples needed to be excluded from the analysis due to the lack of appropriate detection sensitivity in the case of Mandel et al.[20]" in the Discussion section to emphasize that the assay they used did not have appropriate sensitivity.
- We remove parts of the Discussion section about "exerkine" and modified the section so that it clarifies the intention of this article.

Any further responses from the reviewers can be found at the end of the article

Introduction

The role of Brain Derived Neurotrophic Factor (BDNF) in the function of the nervous system is well-established and a large body of work, primarily using mouse models, has long demonstrated this role to be essential for key aspects of brain function.[1] In humans, this essential role is supported by genetic studies including gene deletion and polymorphisms (for a recent review see Ref. 2). BDNF is also likely to be involved in a number of major neurological and psychiatric conditions including Major Depressive Disorder (MDD),[3] Alzheimer's Disease,[4] Parkinson's Disease,[5] and epilepsy.[6] What is less clear is the degree to which measurements of BDNF levels in accessible body fluids may be used to correlate these levels with brain function and dysfunction. While BDNF levels can be readily measured in human blood using traditional BDNF ELISAs, these levels primarily reflect the content of BDNF in blood platelets.[7,8] Thus, whether or not these levels are informative with regard to brain function and dysfunction in humans remains uncertain. This important question has been difficult to answer conclusively because of a major difference between mice and humans with regard to the presence of BDNF in blood. In mice, the most widely used animal model to explore the function and dysfunction of the nervous system, megakaryocytes, the progenitor cells of platelets, do not express the *Bdnf* gene at significant levels, unlike in the case in humans.[8] Very recently, the minute levels of BDNF present in mouse blood could be quantified following the development of a much more sensitive BDNF ELISA.[9] These measurements could also be validated by incubating mouse serum sample with an anti-BDNF monoclonal antibody unrelated to the antibodies used in the BDNF ELISA. While this antibody markedly reduced the ELISA signal, such was not the case when similar experiments were performed with monoclonal antibodies specifically recognizing nerve growth factor (NGF) or neurotrophin-3 (NT3). Both NGF and NT3 are structurally related to BDNF and all 3 bind to the neurotrophin receptor p75 with similar affinities.[10] The BDNF levels determined in mouse blood were found to be about 3 orders of magnitude lower than those determined in human. Unlike is the case with human samples, no difference was found between BDNF levels in mouse plasma or serum, unlike is the case in humans.[9] This finding is consistent with the notion that BDNF in mouse blood does not originate from platelets, but from other sources, including the skeletal musculature as demonstrated by Fulgenzi et al.[11] Very recently, Ikenouchi et al.[12] conducted an independent study using the same BDNF ELISA described in the above and in an attempt to correlate BDNF levels in human saliva with psychological distress in healthcare workers[12] with the values in line with those reported here. Saliva samples can be readily collected and in a large number if needed, with minimum stress even for elderly patients. These straightforward points have attracted the attention of many in the biomedical community in the past as illustrated by a number of reports about measurement of BDNF in human saliva (see for example Zappella et al.,[13] Biamonte et al.,[14] Jasim et al.,[15] and Zhang et al.[16]). However, the BDNF values reported in these studies varied over a very wide range indicating that most BDNF measurement methods used thus far had either not the specificity and/or the sensitivity needed to reliably measure BDNF levels in human saliva, a conclusion already reached by Vrijen et al.[17]

One recent exception was the use of a recently introduced and validated BDNF ELISA kit[9] that was independently used in a study conducted in parallel with ours (Ikenouchi et al.,[12] see Discussion for details).

In the present study, we report the supporting data indicating that the highly sensitive BDNF ELISA assay can be used to accurately measure levels of BDNF in human saliva samples, including after sample storage.

Methods

Samples

Saliva samples were collected from 11 healthy volunteers at the company who did not have known significant health issues or drug usage. Written informed consents were obtained from all of the volunteers according to the company's procedure which is set in accordance with Ethical Guidelines for Medical and Health Research Involving Human Subjects published by Ministry of Health, Labor, and Welfare, Japan. The participants were given a sterilized sample collection tube (2 mL cryovial (Salimetrics, Pennsylvania)) and instructed to rinse their mouth with a cup of water before saliva collection, saliva sample was collected into a sterilized sample collection tube by a passive drool method by using the Saliva Collection Aid (Salimetrics, Pennsylvania) according to the manufacturer's instruction manual. All samples were collected in the same afternoon (noon to 5 pm) and then 6 to 10 mL of the samples were aliquoted into 1.5 mL Eppendorf Safe-Lock Tube (Eppendorf, Tokyo) and then immediately frozen and kept at -80°C until use. At a time of sample use, the samples were thawed at room temperature and then centrifuged at 1500 X g for 15 min to remove remaining particles (pre-treated samples).

Recombinant mature BDNF (PeproTech, Cranbury, New Jersey, USA) was purchased and used as a reference and standard material for the assays.

Measurement of BDNF

BDNF measurement was conducted by using Mature BDNF ELISA kit Wako, High Sensitive (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) according to the instructions provided with the kit. Briefly, samples were diluted 2-fold with the kit Buffer Solution. The BDNF standard stock solution was prepared by adding defined amount of purified water to the Mature BDNF Standard vial, and then a various concentration (0.000, 0.205, 0.512, 1.28, 3.20, 8.00, 20.0, 50.0, and 500 pg/mL) of standard solutions were prepared by diluting the 10 ng/mL Mature BDNF Standard stock solution with Buffer provided in the kit, according to the dilution scheme provided in the instruction. The solution initially filling the ELISA plate was discarded and the wells were washed 4 times with the Wash Solution (1X) included in the kit. The plate was inverted after each wash and gently tapped against clean paper towels to remove any excess liquid retained in the wells. 50 µL of diluted standard solution and of diluted samples were added to respective wells, with duplicate wells used for each standard and sample. After agitating the plate on a microplate mixer, the plate was then covered by Plate Seal (plastic film) and incubated for 2 hours at room temperature (20–25°C). After 2-hour incubation, the solution was discarded, and the wells were washed again 4 times with Wash Solution as above. 50 µL of Biotin-conjugated antibody solution was added to each well, the plate covered by Plate Seal and agitated and then incubated for one hour at room temperature. The solution was then discarded, and wells were washed 4 times with Wash Buffer as above. 50 µL of Peroxidase-conjugated Streptavidin Solution was added to each well, the plate was covered and incubated on for 30 min at room temperature. After a final series of 4 washes with the Wash Buffer, 50 µL of mixed luminescent reagents 1 and 2 (mixed at a ratio of 1:1 before use) were added to each well, the plate was placed on a shaker for 1 min and the luminescence was measured using a 96-well microplate reader Infinite200PRO MPlex from TECAN (Switzerland) at 10 min after the addition of the luminescent reagent. The standard solutions were used to generate a standard curve converting luminescence to BDNF concentrations used to determine the BDNF concentration in the experimental samples. All measurements were conducted twice (n=2) and average of 2 measurements was used for the evaluations.

Results

Spike test

Saliva samples were spiked with 0, 20, or 100 pg/mL of known concentrations of recombinant mBDNF (reference material) at a 9:1 ratio, and then measurements conducted according to the instruction provided in the ELISA kit. The yields of the spiked mBDNF ranged from 85.1 to 102.0%, within the manufacturer's specifications (within ±15%). The distribution of BDNF levels in undiluted samples were 0.296 to 4.09 pg/mL (average = 1.04 pg/mL, SD = 1.09 pg/mL) (Table 1).

Dilution linearity test

Samples were serially diluted using the buffer included in the kit, and BDNF levels measured. As shown in Figure 1, linearity was achieved when at least 2-fold sample dilution (one-to-one dilution) was conducted.

Table 1. Recovery of spiked BDNF in saliva samples.

Saliva sample ID	Spiked BDNF (pg/mL)	Measured BDNF (pg/mL)	Recovered BDNF (pg/mL)	Yield (%)
#1	0	0.783	-	-
	20	18.4	17.7	88.5
	100	90.3	89.6	89.6
#2	0	0.327	-	-
	20	18.2	17.9	89.5
	100	91.6	91.3	91.3
#3	0	1.67	-	-
	20	22.1	20.4	102
	100	98.8	97.1	97.1
#4	0	0.512	-	-
	20	18.1	17.6	88.0
	100	86.9	86.4	86.4
#5	0	0.296	-	-
	20	18.7	18.4	92.0
	100	95.7	95.4	95.4
#6	0	0.393	-	-
	20	17.5	17.1	85.5
	100	85.5	85.1	85.1
#7	0	0.814	-	-
	20	18.4	17.6	88.0
	100	86.4	85.6	85.6
#8	0	4.09	-	-
	20	23.9	19.8	99.0
	100	91.1	87.0	87.0
#9	0	0.601	-	-
	20	18.6	18.0	90.0
	100	101	100	100
#10	0	0.852	-	-
	20	18.3	17.4	87.0
	100	86.3	85.4	85.4
#11	0	1.12	-	-
	20	18.5	17.4	87.0
	100	100	98.9	98.9

Samples were serially diluted by the buffer attached to the commercial kit.

Precision

Samples were spiked with 4 or 40 pg/mL of recombinant mBDNF at a ratio of 9:1. The resulted samples were measured 10 times (n=10) using the ELISA kit to calculate the within precision of the assay. The results showed that intra-assay repeatability was calculated to be within a range of CV =1.8 to 4.9% as shown in Table 2.

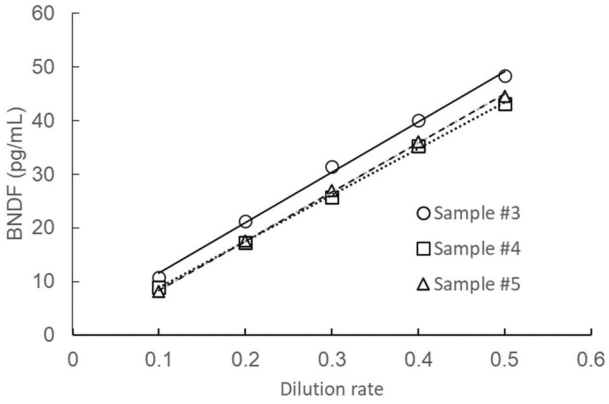

Figure 1. Dilution linearity test.

Table 2. Assay precision of the mBDNF ELISA.

Sample ID	#2		#8		#9	
Spiked BDNF (pg/mL)	4.0	40.0	4.0	40.0	4.0	40.0
n	Measured BDNF (pg/mL)					
1	3.39	36.7	8.05	44.3	3.55	37.1
2	3.40	36.2	8.03	43.5	3.62	37.0
3	3.38	36.4	7.93	44.2	3.41	37.0
4	3.44	35.6	8.20	43.0	3.43	36.5
5	3.24	36.6	8.30	43.3	3.41	38.0
6	3.41	36.7	8.28	43.4	3.61	37.8
7	3.33	36.8	8.48	43.4	3.72	38.2
8	3.27	37.2	8.18	43.3	3.50	37.9
9	3.28	37.3	8.16	44.5	3.60	38.2
10	3.60	39.2	8.23	45.5	3.99	39.3
Mean	3.37	36.9	8.18	43.8	3.58	37.7
SD	0.10	0.95	0.16	0.77	0.18	0.82
CV (%)	3.1	2.6	1.9	1.8	4.9	2.2

Sample stability

Long-term stability

Long term BDNF stability was assessed at 2 different temperatures, -20 and -80°C by using 6 saliva samples spiked with the recombinant mBDNF. They were kept at the designated temperatures for 2 or 3 months, then remaining BDNF levels were measured by the ELISA kit of this study.

As shown in Table 3, degradation of BDNF at -20°C was significant and 30-40% reduction was observed. By contrast, BDNF reduction during 2-month storage was minimal at -80°C and the changes were within 15% although one outlier showed 20% reduction, which was still within the boundary of the acceptable range of 80 to 120% shown in ICH HARMONISED TRIPARTITE GUIDELINE, "Validation of Analytical Procedures: Methodology: Text and Methodology. Q2(R1)" (2005). These data indicates that measurement should be performed within 2 months after saliva sample collection.

Repeated freeze and thawing test

Impact of repeated freeze and thawing was assessed by repeating freeze and thawing cycle of the spiked saliva samples for 5 time and the remaining BDNF levels were measured. As shown in Figure 2, more than 4 times freeze and thawing

Table 3. Long term stability of BDNF in saliva.

a) Stored at -20°C						
ID	**Day 0**		**2 months**		**3 months**	
	BDNF (pg/mL)	**(%)**	**BDNF (pg/mL)**	**(%)**	**BDNF (pg/mL)**	**(%)**
Spiked sample A	1.86	100%	1.17	62.9%	0.46	24.6%
Spiked sample B	3.62	100%	2.23	61.6%	1.77	48.9%
Spiked sample C	5.76	100%	5.71	99.1%	4.05	70.3%
Spiked sample D	18.5	100%	14.2	76.8%	8.00	43.2%
Spiked sample E	29.6	100%	16.9	57.1%	11.3	38.2%
Spiked sample F	37.1	100%	24.0	64.7%	16.8	45.3%
b) Stored at -80°C						
ID	**Day 0**		**2 months**		**3 months**	
	BDNF (pg/mL)	**(%)**	**BDNF (pg/mL)**	**(%)**	**BDNF (pg/mL)**	**(%)**
Spiked sample A	1.86	100%	1.70	91.4%	0.88	47.5%
Spiked sample B	3.62	100%	3.43	94.8%	2.24	61.9%
Spiked sample C	5.76	100%	5.21	90.5%	5.05	87.7%
Spiked sample D	18.5	100%	16.4	88.6%	8.70	47.0%
Spiked sample E	29.6	100%	23.6	79.7%	16.0	54.1%
Spiked sample F	37.1	100%	34.7	93.5%	28.5	76.8%

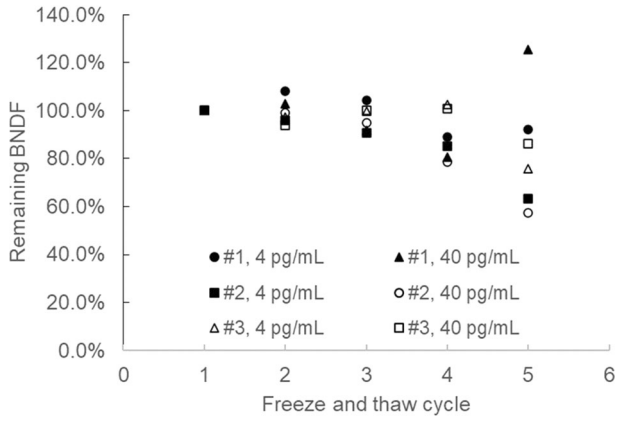

Figure 2. Effect of freeze and thaw cycles for the stability of BDNF in saliva samples.

resulted in 15% or more BDNF reduction, therefore the maximum cycle number applicable to the frozen BDNF samples were determined to be 3 times or less.

Evaluation of sample collection methods

In order to standardize the sample collection procedure, 2 types of saliva collection devices, Salivette made of cotton (Sartstedt, Nümbrecht, Germany) and SalivaBio Infant Swab (Salimetrics, PA, USA) were tested for the mBDNF recovery. The tested devices were soaked with the saliva samples (ID #2, 4, and #11) spiked with known concentrations of recombinant mBDNF and then samples were recovered by centrifugation (1000 X g, 2 min for Salivette and 1500 X g, 15 min for SalivaBio Infant Swab), according to the manufacturers' instructions. The resulted samples were diluted 2-fold and then mBDNF levels were measured. The average recovery of the mBDNF with these commercially available saliva collection devices were 97.8 and 102.3% with the cotton (Salivette) and inactivated polymer (SalivaBio Infant Swab) devices, respectively (Table 4).

Table 4. mBDNF recovery from swab samples.

Saliva sample ID	Spiked BDNF (pg/mL)	Measured BDNF (pg/mL)	Recovered BDNF (pg/mL)	Yield (%)
#2, cotton	0	0.224		
	20	18.6	18.4	92.0
	100	102	102	102
#2, inactivated polymer	0	0.309		
	20	20.5	20.2	101
	100	103	103	103
#4, cotton	0	0.432		
	20	18.2	17.8	89.0
	100	109	109	109
#4, inactivated polymer	0	0.580		
	20	23.0	22.4	112
	100	106	105	105
#11, cotton	0	0.804		
	20	18.0	17.2	86.0
	100	110	109	109
#11, inactivated polymer	0	0.980		
	20	20.9	19.9	99.5
	100	94.1	93.1	93.1

Discussion

The main outcome of this study is that it confirms the notion that BDNF levels can be accurately quantified in human saliva samples with a commercially available, highly sensitive BDNF ELISA kit. The distribution of the BDNF levels in the saliva samples were 0.296 to 4.09 pg/mL, in line with the levels reported by Ikenouchi et al.[12] in a very recent study using the same BDNF ELISA. These levels are indeed extremely low and significantly below the detection limits of other commercially available ELISA kits, in agreement with conclusions reached by Vrijen et al.[17] These levels are also well below the limit of detection of notoriously insensitive methods such as Western blots.[18,19] Also, previous attempts to increase the sensitivity of BDNF ELISA measurements by the use of for example BDNF polyclonal antibodies raise questions about the specificity of such assays.[20,21] Not only do the levels of BDNF in human saliva reported in these previous studies vary greatly but also, they are 2 to 3 orders of magnitude higher than those reported here, i.e. in the sub-ng/mL to ng/mL range (see for example Mandel et al.[20], Bhat et al.[21] , or Tahiroglu et al.[22]), and significant number (up to 35%) of saliva samples needed to be excluded from the analysis due to the lack of appropriate detection sensitivity in the case of Mandel et al.[20]).

Obviously, saliva samples can be readily and repeatedly collected for BDNF level determinations and possible correlations explored with various neurological and psychiatric conditions. While the origin of BDNF in saliva is unclear the study by Ikenouchi et al.[12] demonstrated that there is no correlation between the levels of mBDNF in saliva and plasma. One possible source of BDNF in saliva samples are the sensory nerves innervating the buccal cavity including the tongue and gingiva as the levels of BDNF in sensory afferents are comparatively high (for review, see 2). Given that it is now possible to measure BDNF levels in human saliva, it would be interesting to know whether BDNF levels in saliva correlate with a range of physiological and pathological conditions of interest using appropriate study designs.

Conclusion

The results of this study using a highly sensitive BDNF ELISA demonstrate that saliva samples can be used to measure BDNF levels accurately in readily accessible human samples like saliva, thus confirming the very recent results of Ikenouchi et al.[12] Correlations can now be readily explored between BDNF levels in human saliva and a range of physiological and pathological conditions of interest, including exercise, ageing, depression and neurodegeneration.

Ethical considerations

Collection of the saliva samples from the volunteers was approved at the Expedited 27th Fujifilm Wako Pure Chemicals Life Science Ethics Review Committee with the approval number of #LS 011 on February 3, 2022. The company's Ethics Review Committee was set in accordance with Ethical Guidelines for Medical and Health Research Involving Human Subjects published by Ministry of Health, Labor, and Welfare, Japan, and written informed consents were obtained from all of the volunteers.

Roles

Fumie Akutsu: Data curation; Formal analysis; Investigation; Validation; Writing—original draft; Writing—review & editing. **Shiro Sugino**: Conceptualization; Investigation; Writing—review & editing. **Mitsuo Watanabe**: Writing—review & editing. **Yves-Alain Barde**: Writing—review & editing. **Masaaki Kojima**: Conceptualization; Funding acquisition; Investigation; Supervision; Validation; Writing—review & editing.

Data availability

Dataset for the tables and figures (Saliva validation data for manuscript final.xlsx) is available at https://www.doi.org/10.6084/m9.figshare.27978699.v1.[23]

This project contains the following underlying data:

1. Dataset for the tables and figures (Saliva validation data for manuscript_final.xlsx)

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Publisher Full Text

Open Peer Review

Current Peer Review Status: ✗ ✓ ✓

Version 2

Reviewer Report 28 June 2025

https://doi.org/10.5256/f1000research.181316.r390268

✓ **Sedat Yildiz**
University of Inonu, Malatya, Turkey

I thank to the authors for this simple yet important presentation. As BDNF is associated with many physiological/pathological functions in the body, its non-invasive determination in saliva samples provides a valuable tool for reasearch and developments in associated areas.

The authors have used FUJIFILM Wako's chemiluminescence assay. Although features of this assay is given in the website of the producer, some details need to be given in this manuscript. These are:
1. Please provide reference curve that you have obtained and used for calculation of the results. The readership would like to see the shape of that curve. My imagination, after reading the data in the commercial website, is that the curve is very linear and suitable for determination of low levels of BDNF (such as in saliva).
2. Please provide cross-reactivity of the antibody/test towards the similar molecules such as NGF-beta and NT, as presented in the commercial website. This will help readership to understand specificity of the antibody/test.
3. Please provide the data about the specific recognition of BDNF by the test as revealed by the BDNF knockout mice. This further provides data about reliability of the test developed.
4. Please state (in materials methods section, under mmeasurement of BDNF) thar this test is a chemiluminescence test. So that, the readership can understand it is a sensitive test kit.
5. If it is feasable to you, please provide data that increased saliva level does not result in an interference in the assay. Although dilution study is quite parallel, we need to know matrix effect as the standards are prepared in buffer instead of saliva or artificial saliva. In order to solve this issue, please add increasing amounts of saliva to reference curve and check whether the linearity and parallelism with original standard curve continues. If it is not feasible for you, you can omit this comment.

There are some othe minor corrections that needed to be carried out:
1. In the abstract section, please remove "see discussion" part as it is not necessary in the abstarct
2. In the abstract section, beginf the results with the mean levels of the BDNF, rather than the validation data. Please provide the levels first, then the validation. Because, as a reader, I would

like to see the real/quantitative results in the saliva, as it is said to be very low compared to the blood samples.
3.In the results section, please add the reference curve first, as explained above.
4. In the discussion section, please use a a careful language when referring to the products of the other companies. In that respect, in terms of research oriented kits, no one should expect similar levels accross the products of the different companies. Because, this generally is due to the purity and structure of the test types. But this does not mean that the other test kits are not able to measure the analyte. The level might be different but the measurement might be more accurate. As far as I understand from the producer's web site, this kit is not a "diagnostically approved kit". Therefore, "different levels accross the companies" should not be the backbone of the discussion section. Rather, the usability of these test kits might be discussed or the areas where all these test kits might be used should be explained more.
4.In the first sentence of the discussion, rather than using "confirm", please use "provides evidence" as there should be much more comprehensive studies that the data presented in the current study.
5. In the discussion section, please remove the term "notoriously" as it is not an academic explanation. Please use more direct language like "... detection by the methods like Western blots".
6. Please use a careful language for the test kits that use polyclonal antibodies. Because, in ELISA, sometimes the best results are obtained by the use of polyclonal antibodies or cocktail of the monoclonal antibodies.
7. In conclusions section, please remove "...thus confirming ...". There is no need for the last part of this sentence. There should not be a "confirming" situation taking into account the variables in the test systems and biology itself.
8. Please remove the words "readily" from the conclusion section. Instead, the terms "easily" or "non-invasively" should be preferred.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Immunoassay development, stress physiology, antibody production, assay validation, antibody or antigen tagging with enzymes, immunoaffinity purification,

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 09 June 2025

https://doi.org/10.5256/f1000research.181316.r386656

Bijorn Omar Balzamino
IRCCS Fondazione Bietti, Rome, Italy

The author claims that other kits show very variable values of bdnf in saliva, according to him, very high and therefore influenced by various factors, including the non-specificity of the kit used.
I appreciate the author's response, but I still can't understand how he can justify that this product is more specific than an R&D mab248 duo-set for example. Any kit used for research has a certificate of specificity of the marker, specifying no cross-reactivity with other similar markers. if the author then wants to make this kit usable in diagnostics, the results must be demonstrated differently.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Research and development laboratory for biochemical, molecular and cellular

applications in ophthalmological sciences

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Reviewer Report 06 June 2025

https://doi.org/10.5256/f1000research.181316.r386655

Elizabeth A Thomas
University of California Irvine, Irvine, CA, USA

I have no further comments.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: biomarkers, peripheral biofluids (plasma, saliva), neurodegenerative diseases

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Reviewer Report 04 April 2025

https://doi.org/10.5256/f1000research.176178.r371741

?

Elizabeth A Thomas
[1] University of California Irvine, Irvine, CA, USA
[2] University of California Irvine, Irvine, CA, USA

The paper “A highly sensitive enzyme-linked immunosorbent assay allows accurate measurements of brain-derived neurotrophic factor levels in human saliva” presents important assay details for quantifying BDNF levels in saliva samples. These include spike and recovery data for the saliva matrix, the effects of different saliva collection protocols, linearity of dilution in the saliva matrix, precision and reproducibility of the BDNF measures and information regarding sample stability under long-term storage and the effects of multiple freeze thaws on BDNF detection. These are all essential considerations to show that saliva can be used for BDNF measurements. Saliva is a non-invasive biofluid that has gained recent attention for use in a wide range of human studies; hence knowing assay specifics for quantification of a very popular physiological peptide by ELISA has important consequences for others wanting to measure BDNF in saliva.

The authors list previous studies that have quantified BDNF in human saliva samples and state that “BDNF values reported in these studies varied over a very wide range, indicating that most BDNF measurement methods used thus far had either not the specificity and/or the sensitivity needed to reliably measure BDNF levels in human saliva”. Aside from using different ELISA assays, it is also very likely that these past studies used different saliva collection methods and storage conditions, which the authors show can affect BDNF levels in saliva. Hence, the current findings could possibly explain some of the variability observed in past studies. This is another relevant aspect of the work.

A few other corrections/suggestions:
-In the Introduction, they include Nakagawa et al. (ref 12) and Kikuchi et al., (ref 13) as human saliva studies, when these were carried out in rats. These should be omitted, but there are several other studies that have measured BDNF in saliva samples in humans that could be mentioned.

-In particular, one past study (Mandel A et a., 2011) also had worked out optimized conditions for another BDNF ELISA (although not as in depth as the current paper) and this study should be mentioned somewhere.

-In the Abstract, it is not necessary to mention the mouse study: “Recently, the suitability of a highly sensitive BDNF ELISA assay was validated using mouse plasma and serum where conventional BDNF ELISA fail to detect BDNF”. The fact that it was validated in mouse is not as important as the fact that the assay works well in human saliva.

-The introductory text regarding the origin of plasma/serum-derived BDNF levels is interesting, as

the author make the suggestion that BDNF levels measures in blood might not inform about brain dysfunction in humans. Two previous studies (Ikenouchi A et al., 2023 and Gutierrez A et al., 2020). shown that saliva and plasma levels of BDNF are not correlated, possibly suggesting that saliva measures could be more relevant. This is worth mentioning in the Discussion.

-The time frame (i.e. 2 pm to 5 pm) of saliva sample collection should be stated. Also, the amount of saliva (i.e. 1 ml) collected should be stated.

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: biomarkers, peripheral biofluids (plasma, saliva), neurodegenerative diseases

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 29 Apr 2025

Fumie Akutsu

Dear Dr. Thomas,

Please find our responses written below.

Reviewer's comments:
The paper "A highly sensitive enzyme-linked immunosorbent assay allows accurate measurements of brain-derived neurotrophic factor levels in human saliva" presents important assay details for quantifying BDNF levels in saliva samples. These include spike and recovery data for the saliva matrix, the effects of different saliva collection protocols,

linearity of dilution in the saliva matrix, precision and reproducibility of the BDNF measures and information regarding sample stability under long-term storage and the effects of multiple freeze thaws on BDNF detection. These are all essential considerations to show that saliva can be used for BDNF measurements. Saliva is a non-invasive biofluid that has gained recent attention for use in a wide range of human studies; hence knowing assay specifics for quantification of a very popular physiological peptide by ELISA has important consequences for others wanting to measure BDNF in saliva.

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A few other corrections/suggestions:
-In the Introduction, they include Nakagawa et al. (ref 12) and Kikuchi et al., (ref 13) as human saliva studies, when these were carried out in rats. These should be omitted, but there are several other studies that have measured BDNF in saliva samples in humans that could be mentioned.

1. We agree to your comment to remove Nakagawa et al (ref 12) and Kikuchi et al (ref 13), since their studies are not for human but for rat saliva BDNF levels.

We included the Tahiroglu et al. in the Discussion section (new ref 22) as the most recent report discussing measurement of salivary BDNF.

-In particular, one past study (Mandel A et al., 2011) also had worked out optimized conditions for another BDNF ELISA (although not as in depth as the current paper) and this study should be mentioned somewhere.

2. We add some description about Mandel A et al in Discussion: "and significant number (up to 35%) of saliva samples needed to be excluded from the analysis due to the lack of appropriate detection sensitivity in the case of Mandel et al [20]."

-In the Abstract, it is not necessary to mention the mouse study: "Recently, the suitability of a highly sensitive BDNF ELISA assay was validated using mouse plasma and serum where conventional BDNF ELISA fail to detect BDNF". The fact that it was validated in mouse is not as important as the fact that the assay works well in human saliva.

3. Although the reviewer suggests removing the sentence about the mouse study in the Abstract, we believe this is an important improvement in measurement of BDNF in various samples which was not done by commercially available BDNF assays in the past. So, we

would like to keep the sentence in the Abstract as it specifically refers to validation, an all-important aspect. Sensitivity is of course key but so is validation. Readers may be interested to go back to the study we cite in our manuscript and check for themselves what is meant by validation. Briefly, beyond the reduction of the BDNF signal using a BDNF monoclonal antibody not used in the BDNF ELISA. the finding that BDNF levels in mouse plasma and serum are indistinguishable is key. Indeed, the process of platelet degranulation leads to the release of a number of cytokines and growth factors from platelets and the finding that the BDNF values remain unchanged compared with the plasma values is an important aspect of the validation.

-The introductory text regarding the origin of plasma/serum-derived BDNF levels is interesting, as the author make the suggestion that BDNF levels measures in blood might not inform about brain dysfunction in humans. Two previous studies (Ikenouchi A et al., 2023 and Gutierrez A et al., 2020). shown that saliva and plasma levels of BDNF are not correlated, possibly suggesting that saliva measures could be more relevant. This is worth mentioning in the Discussion.

4. Per reviewer's suggestion, we modify the discussion section: "Obviously, saliva samples can be readily and repeatedly collected for BDNF level determinations and possible correlations explored with various neurological and psychiatric conditions. While the origin of BDNF in saliva is unclear, the study by Ikenouchi et al. [12] demonstrated that there is no correlation between the levels of mBDNF in saliva and plasma. One possible source of BDNF in saliva samples are the sensory nerves innervating the buccal cavity including the tongue and gingiva as the levels of BDNF in sensory afferents are comparatively high (for review, see 2). Given that it is now possible to measure BDNF levels in human saliva, it would be interesting to know whether BDNF levels in saliva correlate with a range of physiological and pathological conditions of interest using appropriate study designs."

-The time frame (i.e. 2 pm to 5 pm) of saliva sample collection should be stated. Also, the amount of saliva (i.e. 1 ml) collected should be stated.
Is the work clearly and accurately presented and does it cite the current literature?

5. We modify the part about the sample collection by adding the time frame and amount of the saliva sample collection to the original manuscript: All samples were collected in the same afternoon (noon to 5 pm) and then 6 to 10 mL of the samples were aliquoted into 1.5 mL

Competing Interests: FA, MW, SS, and MK are employees of FUJIFILM Wako Pure Chemical Corporation. YB is a consultant of FUJIFILM Wako Pure Chemical Corporation

Reviewer Report 24 February 2025

https://doi.org/10.5256/f1000research.176178.r367703

Bijorn Omar Balzamino
[1] IRCCS Fondazione Bietti, Rome, Italy
[2] IRCCS Fondazione Bietti, Rome, Italy

Report

1. **Importance of the idea**.
   - In this short paper the authors analyze the effectiveness of a new ELISA kit for BDNF, using saliva as sample.
   - Unfortunately, the idea is not new and there are several kits on the market for the analysis of BDNF from, which have also been used for the study of BDNF in saliva (see Nakagawa et al., 2019; Ye et al., 2020, Jasim et al., 2020; Kikuchi et al., 2020; Zappella et al., 2021; Biamonte et al., 2022 and Zhang et al., 2024) for example.
   - The method section is well written so for statistical results.
2. **Recommending a Revision and Resubmission:**
   - I think that the entire work should be set up differently, specifying better how other kits already exist on the market and how the BDNF has already been evaluated with these kits, therefore it's more likely to describe a comparison between them.
   - The results and discussion are well written and justified, the only thing that is not well illustrated is the abstract part where it is said that BDNF is not detectable in saliva, and this, as previously stated, is not true.

References

1. Nakagawa Y, To M, Saruta J, Yamamoto Y, et al.: Effect of social isolation stress on saliva BDNF in rat.*J Oral Sci*. 2019; **61** (4): 516-520 PubMed Abstract | Publisher Full Text
2. Ye D, Gajendra S, Lawyer G, Jadeja N, et al.: Inflammatory biomarkers and growth factors in saliva and gingival crevicular fluid of e-cigarette users, cigarette smokers, and dual smokers: A pilot study.*J Periodontol*. 2020; **91** (10): 1274-1283 PubMed Abstract | Publisher Full Text
3. Jasim H, Ghafouri B, Carlsson A, Hedenberg-Magnusson B, et al.: Daytime changes of salivary biomarkers involved in pain.*J Oral Rehabil*. 2020; **47** (7): 843-850 PubMed Abstract | Publisher Full Text
4. Kikuchi T, Sakaguchi W, Saruta J, Yamamoto Y, et al.: Hypertriglyceridemia-induced brain-derived neurotrophic factor in rat submandibular glands.*J Oral Biosci*. 2020; **62** (4): 327-335 PubMed Abstract | Publisher Full Text
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Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Partly

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Author Response 26 Feb 2025

Fumie Akutsu

Dear reviewer,

*"We never claimed that measuring BDNF levels in human saliva is a new idea. Our intention was instead to demonstrate that this is a feasible objective using a BDNF ELISA kit that has the necessary **sensitivity** and **specificity**. Our study confirmed the results of just one previous, recent study using the same BDNF ELISA kit by Ikenouchi and colleagues. This kit is the one BDNF ELISA kit that has been fully validated in a previous study (PMID: 37173369) in which Want and colleagues thoroughly documents both the specificity and sensitivity of this ELISA kit. The other BDNF ELISA kits mentioned by the reviewer report values that are implausibly high for BDNF levels in human saliva, with no demonstration of specificity. We hope that these additional comments will help clarifying what appears to be a misunderstanding about the objective of our study."*

In order to clarify our intention, we will modify the Conclusion to:

Our results indicate that the BDNF ELISA kit previously validated both with regard to specificity and sensitivity (Want et al.) [9] can be used to accurately determine the minute amounts of BDNF present in human saliva. These results also confirm those recently published by Ikenouchi and colleagues [11] using the very same BDNF ELISA kit. Correlations can now be readily explored between BDNF levels in human saliva and a range of

physiological and pathological conditions of interest, including exercise, ageing, depression and neurodegeneration.

We would appreciate your kind review.

Competing Interests: No competing interests were disclosed.

Author Response 29 Apr 2025

Fumie Akutsu

Dear Dr. Thomas,

Please find our responses written below.

Reviewer's comments:
The paper "A highly sensitive enzyme-linked immunosorbent assay allows accurate measurements of brain-derived neurotrophic factor levels in human saliva" presents important assay details for quantifying BDNF levels in saliva samples. These include spike and recovery data for the saliva matrix, the effects of different saliva collection protocols, linearity of dilution in the saliva matrix, precision and reproducibility of the BDNF measures and information regarding sample stability under long-term storage and the effects of multiple freeze thaws on BDNF detection. These are all essential considerations to show that saliva can be used for BDNF measurements. Saliva is a non-invasive biofluid that has gained recent attention for use in a wide range of human studies; hence knowing assay specifics for quantification of a very popular physiological peptide by ELISA has important consequences for others wanting to measure BDNF in saliva.

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report discussing measurement of salivary BDNF.

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-The introductory text regarding the origin of plasma/serum-derived BDNF levels is interesting, as the author make the suggestion that BDNF levels measures in blood might not inform about brain dysfunction in humans. Two previous studies (Ikenouchi A et al., 2023 and Gutierrez A et al., 2020). shown that saliva and plasma levels of BDNF are not correlated, possibly suggesting that saliva measures could be more relevant. This is worth mentioning in the Discussion.

à Per reviewer’s suggestion, we modify the discussion section: “Obviously, saliva samples can be readily and repeatedly collected for BDNF level determinations and possible correlations explored with various neurological and psychiatric conditions. While the origin of BDNF in saliva is unclear, the study by Ikenouchi et al. [12] demonstrated that there is no correlation between the levels of mBDNF in saliva and plasma. One possible source of BDNF in saliva samples are the sensory nerves innervating the buccal cavity including the tongue and gingiva as the levels of BDNF in sensory afferents are comparatively high (for review, see 2). Given that it is now possible to measure BDNF levels in human saliva, it would be interesting to know whether BDNF levels in saliva correlate with a range of physiological

and pathological conditions of interest using appropriate study designs."

-The time frame (i.e. 2 pm to 5 pm) of saliva sample collection should be stated. Also, the amount of saliva (i.e. 1 ml) collected should be stated.
Is the work clearly and accurately presented and does it cite the current literature?

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Competing Interests: No competing interests were disclosed.