biosensis® GDNF *Rapid*™ ELISA Kit: Rat

Catalogue Number: BEK-2230-1P/2P

For the quantitative determination of rat GDNF in cell culture supernatants, cell lysates and serum only if used as directed.

Please refer to the Sample Preparation Section for specific use instructions for each substrate application, in particular rat serum samples.

For research use only, not for use in clinical and diagnostic procedures.

Table of Contents

1. Intended Use

The purpose of this kit is the quantitative determination of rat GDNF in cell culture supernatants, cell lysates and serum only if used as directed. This kit has not been tested for other sample applications. Non-validated samples may contain immunoglobulins and other components that potentially interfere with this ELISA assay. Biosensis does not assume responsibility if this kit is used for unintended purposes.

This ELISA kit is designed to measure the rat form of GDNF. For accurate quantification of GDNF in human and mouse samples, we recommend BEK-2222 and BEK-2229, which use human GDNF and mouse GDNF protein standards, respectively! While all GDNF *Rapid*™ ELISA kits use the same capture and detection antibodies, the protein standards differ to reflect their use in either human or rodent samples.

The assay antibodies cross-react with guinea pig GDNF as evidenced by measuring GDNF in guinea pig serum with the Human GDNF *Rapid*™ ELISA kit (BEK-2222). All Biosensis GDNF *Rapid*™ ELISA kits use the same capture and detection antibodies, and thus this Rat GDNF *Rapid*™ ELISA kit (BEK-2230) can be used to assay guinea pig GDNF in serum. However, in absence of a true guinea pig GDNF protein standard, the Human GDNF *Rapid*™ ELISA kit (BEK-2222) may give the most accurate estimations of guinea pig GDNF levels based on amino acid sequence homology.

For research use only. Not for diagnostic and clinical purposes.

2. Introduction

Glial cell line-derived neurotrophic factor (GDNF) is a 30 kDa homodimer which belongs to the transforming growth factor-β (TGF-β) superfamily. GDNF has pronounced protective effects on midbrain dopaminergic and motor neurons and has been implicated in multiple neurodegenerative diseases including Parkinson's, Alzheimer's and Motor Neuron Disease. Administration of GDNF is considered a potential therapeutic and its effect is investigated in a number or clinical trials.

The Biosensis GDNF *Rapid*™ enzyme-linked immunosorbent assay (ELISA) Kit is a sandwich ELISA that allows the quantification of GDNF in less than 4 hours. This kit consists of a pre-coated anti-GDNF capture antibody, a biotinylated anti-GDNF detection antibody and horseradish peroxidase (HRP)-conjugated streptavidin. The addition of a substrate (3,3',5,5'-tetramethylbenzidine, TMB) yields a colored reaction product which is directly proportional to the concentration of GDNF present in samples and protein standards.

The provided Quality Control (QC) sample serves as a positive control with a defined concentration range of GDNF protein, formulated in a stabilized buffer solution and designed to assure assay performance.

This GDNF ELISA kit employs a recombinant rat GDNF standard and is therefore designed to measure the rat form of GDNF. Note that the antibodies used in this kit cross-react with mouse and human GDNF.

3. Materials Provided and Storage Conditions

Reagent	Quantity	
	1 Plate Kit	2 Plate Kit
GDNF antibody coated 96 well microplate	12 x 8 wells	24 x 8 wells
Assay diluent A (1x)	2 x 25 mL	4 x 25 mL
Recombinant rat GDNF standard	2 x 1000 pg	4 x 1000 pg
Quality Control (QC) Sample	2 Vials (see vial label for amount)	4 Vials (see vial label for amount
GDNF detection antibody (100x)	1 x 110 µL	2 x 110 µL
Streptavidin-HRP (100x)	1 x 110 µL	2 x 110 µL
Wash buffer (10x)	1 x 33 mL	2 x 33 mL
TMB substrate (1x)	1 x 11 mL	2 x 11 mL
TMB stop solution (1x)	1 x 11 mL	2 x 11 mL
Plate sealer	Supplied	

Reagent	Storage and Stability
ELISA kit as supplied & unopened	12 months at 2-8°C
Reconstituted standard and QC sample	Use on same day; aliquot remaining standard to prevent multiple freeze-thaw cycles and store at -20°C for up to 2 weeks
Diluted detection antibody and HRP conjugate (1x)	2 weeks at 2-8°C
Diluted wash buffer (1x)	2 weeks at 2-8°C

Note:

- Do not freeze the streptavidin-HRP conjugate
- Do not use assay diluents from other ELISA kits

4. Equipment Required but Not Supplied

- Single- and multi-channel pipettes capable of delivering 10-1000 µL volumes
- Plastic and glass ware for sample collection, sample preparation and buffer preparation
- Plate shaker
- Microplate reader capable of reading absorbance at 450 nm

5. Before You Start….

- Read the entire protocol to familiarize yourself with the assay procedure; a tutorial about the use of *Rapid*™ ELISA kits is available online at: https://www.youtube.com/watch?v=7EOuc9qYL0E
- Bring the microplate and all reagents and solutions to room temperature before starting the assay
- Remove only the required amount of TMB substrate from the fridge to prevent multiple warm-ups and cool downs; keep the TMB substrate in the dark
- Remove the number of strips required and return unused strips to the pack and reseal
- Centrifuge all kit components to collect reagents to the bottom of tubes before use
- The stop solution provided with this kit is an acid; wear protective equipment when handling
- Please visit our Technical Notes section at www.biosensis.com for further information and helpful hints on ELISA-related topics

Careful experimental planning is required to determine the total number of samples that can be assayed per plate. Refer to Appendix A for help in determining the number of microplates required to conduct an ELISA assay experiment.

6. Sample Preparation

The assay diluent provided in this kit is suitable for measuring GDNF in cell culture supernatants, serum and cell extracts. See the following sample preparation guidelines for specific substrate preparation recommendations and sample dilution instructions. Final working pH of any assay sample should be near neutral (pH 6.8-7.5) for best results, adjust with mild acid or base as needed.

For unknown concentrations of GDNF in samples, it is important to perform several dilutions of the sample to allow the GDNF concentration to fall within the range of the GDNF standard curve (7.8-500 pg/mL). For unknown concentrations of GDNF in samples, it is important to perform several dilutions of the sample to allow the GDNF concentration to fall within the range of the GDNF standard curve (7.8-500 pg/mL). Also, with unknown samples of all types it is highly recommended to run some validation experiments. This should include dilutional linearity/parallelism experiments, and spike- and recovery control tests at least at the minimum required dilution (MRD) for each sample. Parallelism and linearity of diluted samples with the standard curve demonstrates accurate quantification, as well as 80-120% recovery of spiked GDNF. Failure of these essential validation experiments indicates that sample buffer, preparation and dilution and or blocking procedures will need to be optimized by the end user for consistent, accurate results with this ELISA assay. Please refer to our Technical Note #1 (ELISA Assay Validation) for further details.

Cell Culture Supernatants

- Remove particulates by centrifugation (10,000 x g for 5 minutes)
- Analyze immediately or store at -20°C to -80°C in aliquots to prevent multiple freeze thaw cycles
- Samples with high FBS or proteinaceous content will require to be diluted with plain culture medium and/or assay buffer for best results. Also note, bovine GDNF shares high sequence homology with human, mouse and rat GDNF and may be detected in this assay, thus appropriate serum free, cell-free controls must be used for accurate detection.
- Levels of GDNF may be low in standard culture conditions; concentrating cell culture supernatants with ultrafiltration spin columns of ≤ 10 kDa molecular weight cut-off (MWCO) may help to detect low amounts of GDNF
- Protease inhibitors may be added to the sample if enzymatic degradation of GDNF is suspected

Cell Lysates

The antibodies used in this assay are compatible with RIPA buffer. Actual user preparations and testing procedures must be optimized for experimental conditions and cell lines. It is expected that sample lysates will need to be diluted with assay buffer at least 1:5 prior to running the assay with RIPA buffers. Higher dilutions may perform more consistently than lower dilutions.

A general guideline for preparing RIPA extracts is given below:

- Harvest whole cells from cell culture flask after washing off cell culture medium with ice-cold PBS
- Resuspend cell pellet in ice-cold RIPA buffer (50 mM Tris, 150 mM sodium chloride, 2 mM EDTA, 1% NP-40 or Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, pH 7.4-8.0); do not add reducing agents such as DTT or β-mercaptoethanol
- The addition of a protease inhibitor cocktail to RIPA buffer just prior to cell lysis is highly recommended
- Lyse the cells on ice for 30 minutes
- Centrifuge cells for 15 minutes at 21,000 x g at 2-8°C
- Measure total protein concentration (eg., BCA or Bradford protein assay)
- Aliquot the supernatant into useful aliquots and store at -80°C; prevent multiple freeze-thaw cycles

As an alternative, freeze whole cell pellets and store at -80°C for cell lysis at later time-point.

Serum

- Allow the serum to clot in a serum separator tube (about 30 minutes to 4 hours) at room temperature
- Centrifuge at approximately 1,000 x g for 15 minutes
- Analyze the serum immediately or aliquot and store frozen at -20°C to -80°C
- Dilute serum samples with Assay Diluent A in order to measure GDNF concentrations; limited data on rat serum indicates a minimum required dilution of 1:2 to achieve acceptable recoveries

Important: Expected endogenous GDNF concentrations in normal rodent blood are very low and sensitivity of this ELISA assay may not be sufficient.

Acid Treatment of Samples

Studies have shown that GDNF is bound to receptors in tissue preparations masking it from detection by any ELISA method. Acid-treatment of tissues has been demonstrated to be effective in releasing GDNF from its receptors. A simple acid extraction method is summarized here based on published work by Okragly A.J. and Haak-Frendscho M. (1997).

- Dilute a portion the primary sample 1/4 to 1/5 in a simple buffer such as Hank's or PBS; use undiluted sample for very low abundance of GDNF
- Acidify this sample aliquot with 1N HCl to a pH of about 3, but avoid acidification to values lower than pH 2
- Spot a small aliquot of the solution on pH paper to confirm acidification
- Incubate at room temperature for 10-15 minutes
- Neutralize to pH 7.0-7.6 and confirm neutralization with pH paper
- Store acid-treated samples in aliquots at -80°C for no more than 6 months and avoid freeze-thaw cycles

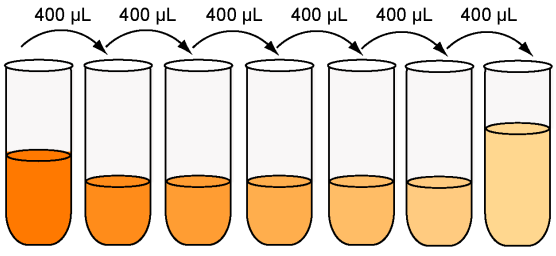
Note: These sample preparation methods are given as a guideline and may need to be optimized and adapted to specific experimental conditions!

7. Preparation of GDNF Standard

- Reconstitute the lyophilized antigen standard with 1 mL of the **same assay diluent used for preparing sample dilutions**
- Label the vial with the reconstituted GDNF standard as “1000 pg/mL”; vortex and let stand for 15 minutes
- Dilute the 1000 pg/mL GDNF standard 1:2 (eg., 500 µL of 1000 pg/mL standard + 500 µL assay diluent); label this tube “500 pg/mL”
- **Note:** 500 pg/mL is the highest concentration of the GDNF standard curve

In order to generate a GDNF standard curve, perform a 1:2 serial dilution down to 7.8 pg/mL. The volumes used for the dilution series depends on the number of repeats per GDNF concentration. For triplicate measurement (100 µL per well) of each GDNF standard concentration, we recommend this procedure:

1. Label 6 tubes with “250 pg/mL”, “125 pg/mL”, “62.5 pg/mL”, “31.3 pg/mL”, “15.6 pg/mL” and “7.8 pg/mL”, respectively
2. Aliquot 400 µL of the assay diluent into each tube
3. Take 400 µL from the “500 pg/mL” tube and transfer to the “250 pg/mL” tube
4. Pipet up- and down and vortex to mix; to avoid foaming, use only a very brief vortex
5. Repeat steps 3 and 4 for each consecutive concentration until the last tube “7.8 pg/mL” is prepared and mixed well



GDNF (pg/mL)	500	250	125	62.5	31.3	15.6	7.8
Final volume in vial (µL)	600	400	400	400	400	400	800

8. Other Reagents and Buffer Preparation

- Quality Control (QC) sample: Reconstitute the lyophilized sample with 1 mL of the **same diluent used for preparing the GDNF Standard curve**. This will provide a QC sample within 88 – 163 pg/mL.
- Detection antibody (100x) and streptavidin-HRP conjugate (100x): dilute one hundred-fold with Assay Diluent A; **do not use** culture medium or other buffers and prepare enough volume to add 100 µL per well
- Wash buffer (10x): crystals may form at the bottom of the bottle; equilibrate to room temperature, mix thoroughly and dilute ten-fold with ultrapure water; each 8-well strip requires about 25 mL of 1x wash buffer per assay

9. Assay Procedure

All steps are performed at room temperature (20-25°C, 70-75°F).

1. Add 100 µL of diluted GDNF standards, QC sample, samples and blank (assay diluent only) to the pre-coated microplate wells
2. If available, include a negative and positive control sample in the assay procedure
3. Seal the plate (eg., with plate sealer or parafilm) and incubate the plate on a shaker (140 rpm; 0.351 G*) for 90 minutes
4. Discard the solution inside the wells and perform 5 washes with 1x wash buffer (200 µL per well). See the technical hints section for a detailed description of the washing procedure
5. Add 100 µL of the detection antibody (1x) into each well
6. Seal the plate (eg., with plate sealer or parafilm) and incubate the plate on a shaker (140 rpm; 0.351 G*) for 60 minutes
7. Discard the solution inside the wells and wash as described in step 4
8. Add 100 µL of the 1x streptavidin-HRP conjugate into each well
9. Seal the plate (eg., with plate sealer or parafilm) and incubate the plate on a shaker (140 rpm; 0.351 G*) for 30 minutes
10. Discard the solution inside the wells and wash as described in step 4
11. Add 100 µL of TMB into each well and incubate plate at room temperature for 10-20 minutes without shaking in the dark
12. Stop the reaction by adding 100 µL of the stop solution into each well. Visible blue color will change

to yellow. Read the absorbance at 450 nm on a plate reader. **Note:** Color will fade over time; hence, we recommend plate to be read within 5 minutes after adding the stop solution or no longer than 30 minutes after addition

13. See Appendix B for a troubleshooting guide when unexpected difficulties are encountered

* RCF= 1.12 x Orbit Radius x $(rpm/1000)^2$

10. Technical Hints

1. Do not perform dilutions within the well
2. At least duplicate measurements for each standard and sample dilution is recommended
3. Dilute samples to a GDNF concentration that falls within the range of the standard curve. Do not extrapolate absorbance readings
4. Avoid touching the inside surface of the wells with the pipette tip
5. Proper emptying and washing the plate is crucial for low background and non-specific binding. For manual plate washing, we recommend the following procedure:
    a. To remove liquid from the wells, place the plate on the palm of the hand and quickly invert the plate over the sink. Forcefully move the arm downwards and stop abruptly to force the liquid out of the wells. When done correctly, this technique should prevent liquid from getting onto the fingers or onto the outside of the microplate wells
    b. Blot and forcefully tap the microplate against clean paper towels for 3-5 times
    c. Wash the wells by pipetting 200 µL of wash buffer into each well and empty the wells as described in step a-b)
    d. Repeat this procedure for a total of 5 times
6. Complete removal of liquid from the wells at each step is essential for reliable results. However, avoid letting the wells dry out at any time
7. Add TMB and the stop solution to the wells in the same order
8. Ensure that all bubbles are removed and that the bottom of the plate is optically clean prior to taking the absorbance reading
9. Stopping the TMB reaction after 10-20 minutes is usually sufficient to obtain a very sensitive standard curve. However, TMB incubation times may vary based on the accuracy of diluting the ELISA kit reagents and differences in incubation time and temperature. We use a plate shaker set to 140 rpm and perform all incubations at room temperature (20-25˚C, 70-75˚F) in our laboratories. Importantly, we read our plates on a microplate reader capable of measuring up to an optical density (OD) of 4. You may need to adjust the TMB incubation time if your microplate reader has a more limited range.
10. TMB incubation times can vary between 10 – 20 minutes, and assay sensitivity can be affected if color development is stopped too early. In order to determine the optimal time to stop the TMB incubation, blue color development should be monitored at **650 nm**. Addition of Stop Solution will convert blue into yellow color, with an ~2.5-3X increase in OD at **450 nm**. Once the highest standard reaches an OD of ~0.9 – 1.1 at 650 nm, immediately stop the reaction by adding Stop Solution. Note that before taking a 650 nm measurement, the plate should be briefly and gently shaken by hand to homogenize the blue TMB reaction product within the wells for most accurate readings.

11. Calculation of Results

1. Average the readings for each GDNF standard concentration, blank and sample
2. Plot a standard curve with the GDNF standard concentration on the x-axis and the OD at 450 nm on the y-axis
3. If values for the GDNF standards are adjusted for background absorbance, then subtract the blank value from the OD_{450} of the samples as well
4. Use appropriate software to reduce the data and generate a four parameter logistic (4-PL) curve-fit; avoid using linear regression analysis
5. Perform a 4-PL regression analysis to calculate the concentration of GDNF in the QC sample. An observed concentration within the range of 88 – 163 pg/mL indicates acceptable assay performance
6. Perform a regression analysis to calculate the concentration of GDNF in the samples. Multiply the result by the sample dilution factor

Manual Plate Reading

The relative OD_{450} = (the OD_{450} of each well) – (the OD_{450} of Zero well).

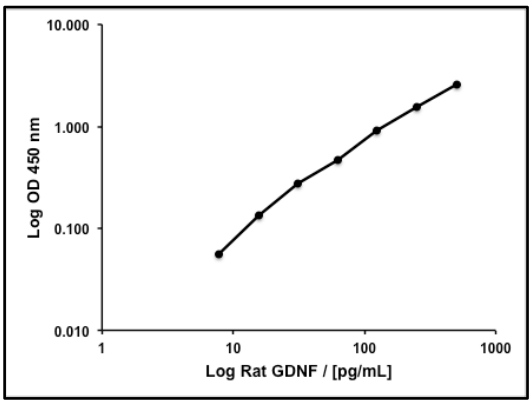
1. The **standard curve** can be plotted as the relative OD_{450} of each GDNF standard solution (Y-axis) vs. the respective known concentration of the GDNF standard solution (X-axis). The standard curve should result in a graph that shows a direct relationship between target protein concentrations and the corresponding OD_{450}. The greater the concentration of target protein in the solution, the higher the OD_{450}
2. **Determine concentration of target protein in unknown sample.** The GDNF protein concentration of the samples can be interpolated from the standard curve. Draw a horizontal line to intersect with the standard curve. A vertical line dropped from this point intersects the X-axis at the concentration of GDNF in the unknown sample. In the exemplary standard curve, a sample with OD_{450} = 0.5 reads as 50 pg/mL GDNF (red line). If the samples measured were diluted, multiply the concentrations from interpolation (see step 1) with the dilution factor to obtain the actual GDNF concentration in the sample

12. Typical Data

Standard Curve

Standard curves are provided for demonstration only. A standard curve has to be generated for each GDNF ELISA assay.

In addition, we recommend measuring common samples when performing multiple assays across several days in order to normalize the standard curve numbers between the various runs.



In the example graph, GDNF standards were run in duplicates using the assay diluent provided in this kit. The TMB reaction was stopped after 15 minutes.

Typical optical densities and coefficient of variations for diluted standards are summarized in the following table:

	OD 450 nm
GDNF / [pg/mL]	Mean
500	2.685
250	1.653
125	1.004
62.5	0.555
31.3	0.363
15.6	0.223
7.8	0.145
Blank	0.089

SD: standard deviation; CV: coefficient of variation

Limit of Detection

This GDNF ELISA kit typically detects a minimum of 5 pg/mL rrGDNF (defined as concentration at Blank OD + 3 x SD_{Blank}, n=10).

Recovery

GDNF protein (200 pg/mL) was spiked into various samples and recoveries determined.

Sample	Dilution	Recovery
C6 Cell Supernatant	1/5	84%
	1/10	119%
	1/20	107%
	1/40	82%
RIPA Buffer	1/5	82%
	1/10	110%
	1/20	102%
	1/40	91%

Endogenous GDNF was not found (< LOD). Note that these samples were not acid-treated.

Recovery in Rat and Guinea Pig Serum

50 pg/mL of GDNF was spiked into dilutions (1:2 to 1:16) of rat and guinea pig serum and recovery of GDNF determined (n = 3 independent assays). Note that guinea pig GDNF levels were quantified against human GDNF protein (BEK-2222).

	Recovery (%)			
Serum Sample	**1:2**	**1:4**	**1:8**	**1:16**
Rat (n=3)	113	94	89	85
Guinea Pig (n=3)	106	104	105	106

Acceptable recoveries are achieved for dilutions of 1:2 and higher for rat and guinea pig serum.

Quantification of GDNF in Rat and Guinea Pig Serum
Serum samples of each rat and guinea pig were assayed on separate days (n = 3 assays) for their GDNF content. Note that guinea pig GDNF levels were quantified against human GDNF protein (BEK-2222).

	GDNF (pg/mL			
Serum Sample	**1:2**	**1:4**	**1:8**	**1:16**
Rat (n=3)	< LOD	< LOD	< LOD	< LOD
Guinea Pig (n=3)	292	314	307	282

No GDNF was detected in rat serum at dilutions of 1:2 to 1:16. In contrast, ~300 pg/mL of GDNF were detected in guinea pig serum. Note that these samples were not acid-treated.

Specificity
The antibodies used in this kit detect mouse and human GDNF. It is expected that this ELISA kit cross-reacts with a number of other species due to a high degree of sequence homology.

No cross-reactivity was observed for the following proteins tested at 25 ng/mL in assay buffer: brain-derived neurotrophic factor (rhBDNF), nerve growth factor (rhNGF), neurotrophin-3 (rhNT-3), rhNT-4/5, vascular endothelial growth factor ($rhVEGF_{165}$), recombinant human Neurturin, Artemin and Persephin.

13. Informational References

Okragly AJ. & Haak-Frenscho M., **An Acid-Treatment Method for the Enhanced Detection of GDNF in Biological Samples.** Exp Neurol. 1997;145, 592-596.

14. Other Information

For accurate quantification of human and mouse GDNF, we recommend the Biosensis GDNF *Rapid*™ ELISA kits which contain rhGDNF (Cat# BEK-2222) and recombinant mouse GDNF standard (Cat# BEK-2229), respectively. Please visit our website (www.biosensis.com) for a full range of GDNF-related research reagents.

Appendix A: Determining the Number of Microplate Wells Needed for ELISA Experiments

Standard curve, blank and controls:

- Standard (500 pg/mL. 250 pg/mL, 125 pg/mL, 62.5 pg/mL, 31.3 pg/mL, 15.6 pg/mL, 7.8 pg/mL): 7 wells
- Blank (0 pg/mL): 1 well
- Control vial (either provided in the ELISA kit or the researcher): 1 well

Gives a total of 9 wells used per Standard/Control set.

We recommend that the standard curve, blanks, and controls be run on each separate plate for the most accurate calculations. Note that if two (or more) independent assays are run on one plate, each assay requires its own Standard/Control set. We also recommend that ALL samples (i.e. standards, controls, blanks and test samples) be performed in duplicate at least.

Thus, for standards, blanks and controls, 9 x 2 = **18 standard wells are required per assay.** This leaves 96 wells – 18 wells = **78 sample wells per plate** for test samples for EACH of our standard 1P ELISA kits run with a standard curve and controls.

1P kit: 78 experimental wells per 96 well ELISA plate experiment
2P kit: 156 experimental wells per 192 well ELISA plate experiment

The 2-Plate Optional Single Control Set:
Some researchers using our 2P kits run a single, duplicate standard curve, blank and control sample set (18 wells) for the entire 2-plate experiment. This allows 78 test samples on the "standard curve plate" (the plate containing the standard curve, controls and blanks) and a full 96 test samples on the "test only plate" for a total available number of 174 tests per 2 plate kit. (78 wells +96 wells = 174).

2P kit, single set of controls (in duplicate): 174 experimental wells per 192 well ELISA plate experiment

Example: 60 test samples, duplicate measurement at one single dilution
How many wells and plates do I need for sixty test samples at one dilution with a single sample taken before and after treatment?

1. Calculate the number of test samples: 60 samples, x 2 draws each (e.g. before and after) = 120 stock samples
2. Factor in the number of repeat tests desired, in order to calculate the number of wells required. Biosensis recommends that ALL samples be run in duplicate at least.
3. Multiply the number of stock samples by the number of dilutions per sample. In this example, we will be testing EACH stock sample at a single dilution (i.e. 1:100) in duplicate, so 120 stock samples x 2 wells = 240 microplate wells required for sixty test samples. In summary: 60 samples, 4 tests per sample, equals 240 total number of wells required.
4. Decide on the number of standards, blanks and controls run on each plate. We recommend standard, control and blank duplicates for each 96-well plate. Alternatively, one standard, blank and control duplicate set can be run on every 2 plates (2-Plate Optional Single Control Set option discussed above).

Running the Test:
Running the 60 test sample experiment with a standard curve, blanks and controls on *each* test plate, in duplicate (our recommended option):

Single Dilution per Test Sample:

(240 wells required) / (78 wells per plate available) = 3.077 plates required, or just over 3 plates, thus researcher will need to order **4 x 1-plate kits or 2 x 2-plate kits** (there will be unused wells) to ensure enough wells for the entire sixty test samples, tested in duplicate (two draws per sample, 1 dilution, 4 wells per test, total of 240 wells). The unused 8-well strips can be used for other assays later.

Two Dilutions per Test Sample:

If testing at two dilutions, (e.g. 1:50 & 1:100 per sample) and doing duplicates, then the number of wells/tests is doubled to 480 (e.g. 120 x 2 @ 1:50 = 240, PLUS 120 x 2 @1:100 = 480). Then the number of plates is determined by (480 wells required) / (78 wells per plate available) = 6.15 plates required, or just over 6 plates, thus the researcher will need to order **3 x 2-plate kits and 1 x 1-plate kit** to ensure the minimum number of wells for sixty test samples tested in duplicate at two dilutions (two draws per test, 2 dilutions, 8 tests/wells per test, total of 480 tests/wells).

2-Plate Optional Single Control Set Option:

If running 60 test samples with a single dilution, and a single duplicate standard curve, blank, and control sample for every two plates, then one has **174 available test wells per 2P kit**.

For the single dilution, sixty test samples, 2 draws per sample experiment (240 tests), one would need (240/174) = 1.38 2-plate kits, or **2 x 2-plate kits** would need to be ordered to ensure enough wells for all sixty test samples.

For the two dilutions per sample, sixty test samples, 2 draws per sample experiment (480 tests), one would need (480/174) = 2.76 2-plate kits, or **3 x 2-plate kits** would need to be ordered to ensure enough wells for all sixty test samples.

Appendix B: Troubleshooting Guide

This GDNF ELISA kit has been developed to deliver reproducible results when following the provided assay protocol. Please refer to the troubleshooting guide below when unexpected difficulties are encountered. If you require further assistance, talk to a scientist at Biosensis (biospeak@biosensis.com).

Problem	Cause	Solution
High background (blank OD > 0.30)	Insufficient washing	Make sure to perform 5 washes after each incubation step with sample, detection antibody and streptavidin-HRP conjugate. Follow the recommended manual washing procedure for optimal washing performance
	Excessive concentration of detection antibody and/or HRP-conjugate	Re-examine the calculations performed to dilute the concentrated stock solutions. Verify that the pipettes dispense the correct volume.
	Increased incubation time and temperature	Please follow incubation times as stated in the protocol and perform incubations at room temperature
	Contamination	Ensure that all plastic- and glassware used is clean. Do not use the same pipette tip to transfer samples and blank solution into the wells
Low absorbance readings	Concentration of GDNF in the sample is lower than the detection limit of this assay	Confirm that the assay protocol was carried out correctly by either spiking a known amount of GDNF into your sample and/or check that the QC samples value falls within the expected GDNF concentration range
	Insufficient antibody or insufficient HRP-conjugate	Re-examine the calculations performed to dilute the concentrated stock solutions. Verify that the pipettes dispense the correct volume
	Reagents expired	Ensure correct storage of the kit and do not use the kit beyond its expiry date
Low absorbance readings	Decreased incubation times and temperature	Please follow incubation times as stated in the protocol and warm reagents to room temperature before use
	Microplate wells dried out	Do not leave microplate wells without solution for an extended time
	Wash buffer not diluted	Ensure that the wash buffer is diluted with ultrapure water to a 1x working solution
	Stop solution not added	Add the stop solution to obtain a yellow reaction product that can be measured at 450 nm
Standard OD values above plate reader limit	Excessive incubation with TMB substrate solution	Reduce incubation time by stopping the reaction at an earlier time-point
Sample OD values above standard curve range	GDNF concentration in sample is too high	Perform a serial dilution of your sample to obtain a reading that falls within the standard curve
High coefficient of variations (CV)	TMB and stopping solution not added consistently	Add TMB and stopping solution to the wells in the same order and speed
	Inconsistent pipetting	Ensure pipettes are working correctly and are calibrated; review your pipetting technique; add standards and samples to the wells using a single-channel rather than a multi-channel pipette
	Insufficient mixing of reagents	Briefly vortex to mix solutions before pipetting into the wells
	Bottom of the plate is dirty	Clean the bottom of the plate before reading the plate