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Optimization of BDNF ELISA Method

Metropolia Ammattikorkeakoulu
Bachelors of Laboratory Services
Bachelor Thesis
2.12.2013

Tekijä Otsikko Sivumäärä Aika	Hanna-Kaisa Vikkula BDNF ELISA -menetelmän optimointi 25 sivua + 2 liitettä 2.12.2013
Tutkinto	Laboratorioanalyttikko AMK
Koulutusohjelma	Laboratorioala
Ohjaajat	Tomi Rantamäki, FaT, dosentti Juha Knuuttila, FM, lehtori
<p>Neurotrofiini aivoperäinen hermokasvutekijä (BDNF) osallistuu keskeisellä tavalla hermo-so-lujen suojaamisen, erilaistumisen, ja muovautuvuuden säätelyyn. Monet kansantalou-delli-sesti merkittävät hermosairaudet on liitetty BDNF proteiinin ilmentymisen ja/tai toimin-nan häiriöihin.</p> <p>Professori Eero Castrénin tutkimusryhmä on aiemmin pystyttänyt BDNF proteiinin mittaa-miseen käytettävän ELISA (enzyme-linked immunosorbent assay) menetelmän. Menetel-mä pohjautuu keskeisesti hiiressä tuotettuihin monoklonaalisiin vasta-aineisiin. Tämän opin-näytetyön tavoitteina on ollut tunnistaa uusia vasta-ainepareja käytettäväksi mene-telmässä sekä itse menetelmän validointi. Menetelmä optimoitiin sensitiivisyyden, selektiivisyyden, detektiomenetelmän ja standardinsuoran suhteen.</p> <p>Tutkimuksissa löydettiin erittäin toimiva vasta-ainepari, ja niihin perustuva menetelmä se-lektiivisesti tunnistaa neurotrofiiniperheen kasvutekijöistä ainoastaan BDNF proteiinia. Myös menetelmän herkkyys parani: lopullinen detektoraja ~15,2 pg/μl. Työssä tutkittiin lisäksi värireaktioon eli kolorimetriseen mittaukseen perustuvaan standardimittauksen rinnalla mahdollisuutta käyttää kemiluminesenssiin perustuvaa mittausta. Kyseinen mittaustapa näytti lupaavalta mutta jatkotutkimuksia tarvitaan sen validoimiseksi analyttiseksi mene-telmäksi. Kaiken kaikkiaan opinnäytetyöltä vaadittavat tavoitteet saavutettiin selvästi.</p>	
Avainsanat	BDNF, ELISA, neurotrofiini

Author Title	Hanna-Kaisa Vikkula Optimization of BDNF ELISA
Number of Pages Date	25 pages + 2 appendices 2 December 2013
Degree	Bachelor of Laboratory Services
Degree Programme	Laboratory Sciences
Instructor(s)	Tomi Rantamäki, PhD, Docent Juha E. A. Knuutila, M. Sc, Lecturer
<p>Brain derived neurotrophic factor, BDNF, regulates the survival, differentiation and plasticity of neurons. Emerging evidence suggests that many highly prevalent and disabling nervous system disorders are associated with altered synthesis and functions of BDNF.</p> <p>Eero Castrén's research group has previously set up an ELISA (enzyme linked immunosorbent assay) for the detection of BDNF. The aim of the thesis was to optimize the ELISA method for the detection of brain derived neurotrophic factor (BDNF) with a new antibody combination and validate the optimized method. The method was optimized for sensitivity, selectivity, detection methods and for the standard curve.</p> <p>A new antibody combination was found. The new combination was more sensitive than the previous combination. The new minimum detection limit was ~15 pg/μl. The antibody combination was also specific only to BDNF when tested with other molecules of the same neurotrophin family. The method was based on colorimetric detection.</p> <p>Furthermore, the potential utilization of chemiluminescence based detection method was tested. This detection method showed promising but further studies are needed to validate its use for analytical assay.</p>	
Keywords	BDNF, ELISA, neurotrophin

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Abbreviations

BDNF	Brain-derived neurotropic factor
ELISA	Enzyme-linked immunosorbent assay
NGF	Nerve growth factor
TrkB	Tropomyosin related kinase B
p75 ^(NTR)	Low-affinity nerve growth factor receptor
CNS	Central nervous system
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline with Tween-20
BSA	Bovine serum albumin
HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
NP	Lysis buffer
PCR	Polymerase chain reaction
DNA	Deoxyribonucleic acid
POD	Peroxidase
LOD	Limit of detection
rpm	Rounds per minute
rcf	Relative centrifugal force

Introduction

Neurotrophin BDNF (brain-derived neurotrophic factor) regulates the survival, differentiation and plasticity of neurons. Emerging evidence suggests that many highly prevalent and disabling nervous system disorders are associated with altered synthesis and functions of BDNF. For example reduced BDNF signalling is implicated with the pathophysiology of Alzheimer's disease and depression. Moreover, many clinically used drugs seem to act, at least in part, by increasing the synthesis and functions of BDNF in the brain. Therefore, methods that allow sensitive and specific quantification of BDNF in brain tissues and bodily fluids are important in clinical and experimental research. Enzyme-linked immunosorbent assay (ELISA) is relatively straightforward and sensitive method for the detection of small quantities of proteins and other analytes including BDNF.

Dr. Eero Castrén's laboratory at the Neuroscience Center, University of Helsinki, have previously developed and used BDNF ELISA method to analyse BDNF protein levels in rodent brain tissues and rat and human blood (Karpova et al., 2010, Spulber et al., 2010). The aim of this thesis was to further optimize this method and to set up and utilize new antibody clones for the methodology. Such effort was required as the previous antibodies were running low.

The study firstly introduces the theoretical background of neurotrophins, outlines the ELISA method and illustrates the antibody production of BDNF antibodies. Then the materials and methods were outlined. The results were illustrated after the materials and method. The results were then discussed and the validation plan was drafted. Lastly the conclusions of the study were discussed.

1 Theoretical Background

To appreciate the significance of the brain derived neurotrophic factor the history leading up to its discovery is vital. The discovery of the nerve growth factor lead way to the understanding of the role of neurotrophins in the mammalian nervous system. The neurotrophin family bring on a cascade of changes in the mammalian brain which allow for the development and refinement of the brain.

Nerve growth factor or NGF was discovered by Rita Levi-Montalcini in 1956. Levi-Montalcini's discovery of NGF spurred the other discoveries of the different families of neurotrophins. Her research took place before, during, and after the Second World War.

Born into a Jewish family in 1909, Rita Levi-Montalcini grew up in a well-off intellectual family. Levi-Montalcini was motivated to pursue a career in medicine though it was not common in the era for women to attend education which would interfere with marital and maternal obligations. Already during her schooling she began working in research under histologist Giuseppe Levi, staining nerve cells with the silver staining method developed by Camillo Golgi. The staining method proved to be the key method in her research success. In 1939, due to the anti-Semitic environment, Levi-Montalcini was forced to conduct her research undercover in her secret laboratory she built into her bedroom. She worked with chicken embryos to study the formation of limbs by viewing neuronal development. She noted that neurons still developed and differentiated though a developing limb had been removed. The neurons only started to degenerate after they had reached the stump of the amputation site. In 1942 Levi-Montalcini and her family fled from Turin and moved into a rural cottage where she continued her research, though only for a short time as Mussolini was overthrown by German troops. Levi-Montalcini and her family then fled to Florence until the end of the war. (Zeliadt, 2013)

After the Second World War, Levi-Montalcini resumed working at the Turin University. In 1946, she received a letter from Victor Hamburger, the author of an article that had spurred Levi-Montalcini's research to investigate the neurons involved with limb formation in the chick embryos. She then moved to St. Louis to repeat and expand her findings with Dr. Hamburger in 1947. This move was to last for the next 30 years. Her research moved onto observing the behaviour of tumours implanted into chicken embryos. In her observations she found that the nerve cells did not make direct contact with the tumour cells, so she hypothesised that the tumour secreted a growth promoting factor for the neurons. To determine if the growth-promoting agent was a nucleic acid or protein, a solution of tumour cells and snake venom was used. Snake venom contains phosphodiesterase, an enzyme which degrades nucleic acids. The results of the experiment were opposite to what were expected as the snake venom enhanced the growth promoting effects of the tumours. Stanley Cohen, one of Levi-Montalcini's colleagues, identified the protein that was later named as the NGF. Levi-Montalcini and Cohen started searching for the growth factor in other tissues as the extraction of

the growth factor would have been too expensive and tedious from the snake venom. Cohen serendipitously found that male mouse salivary glands contained a tenfold concentration of the NGF which they were able to purify in large quantities (Zeliadt, 2013).

Since the discovery of NGF the science of growth factors has become vital part in the study of brain and organism development. This thesis concentrates on the brain-derived neurotrophic factor (BDNF).

1.1 BDNF

The following figure depicts the crystalline structure of the Neurotrophin family. It is vital to note the similar structures of the molecules.

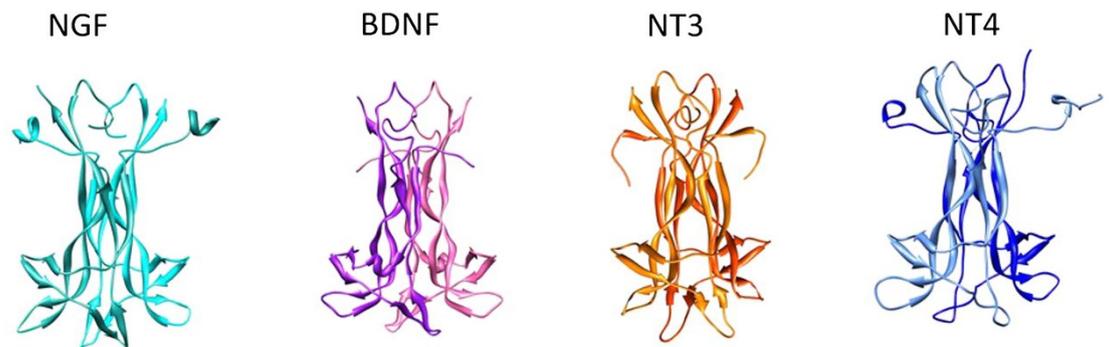


Figure 1. The neurotrophin family. Picture edited from (Allen et al., 2013) with permission from Elsevier

BDNF belongs to the NGF family of neurotrophic factors (including also NT-3 and NT-4/5) and shares about 50% amino acid identity with this neurotrophin family (Binder and Scharfman, 2004). BDNF was discovered by Yves-Alain Barde, David Edgar and Hans Thoenen in the beginning of the 1980's (Barde et al., 1982). After its discovery, numerous studies have been completed in understanding its neurophysiology. Emerging evidence shows that BDNF critically regulates the development and survival of subpopulation of neurons in the peripheral nervous system (Huang and Reichardt, 2001). In the developing and adult central nervous system (CNS), the important role of BDNF in regulating neuronal and synaptic plasticity, neurogenesis and neuronal differentiation has been well characterized. Indeed, abnormal BDNF synthesis and functions has been associated with variety of nervous system disorders including neurodegenerative disorders, anxiety and depression. On the other hand, many clinically used drugs,

particularly antidepressant drugs, seem to act, at least in part, by promoting the expression of BDNF and its signalling in the brain (Castrén and Rantamäki, 2010).

The *Bdnf* gene in humans is found on chromosome 11p, has eleven 5' exons and one 3' exon that encode the mature BDNF protein (Pruunsild et al., 2007). BDNF is initially synthesized as an uncleaved precursor protein, pro-BDNF. Most importantly, pro-BDNF has different binding characteristics and distinct biological activities in comparison with the mature BDNF protein (Teng et al., 2005). The mature-BDNF binds with high affinity to TrkB tyrosine kinase receptors. Upon BDNF binding TrkB receptors are dimerized and autophosphorylated, which further leads to activation of signalling cascades that regulate neurotrophic and neuroplastic changes in the cell. However, the pro-BDNF preferentially binds p75 neurotrophin receptor or also known as low-affinity nerve growth factor receptor (p75^{NTR}), a member of tumor necrosis factor family receptors, and thereby produces opposite changes on neuronal function such as apoptosis. p75^{NTR} is predominantly expressed during the early neuronal development. However, various pathological conditions, including epilepsy, axotomy and neurodegeneration, can induce p75^{NTR} expression in the adult brain.

The amino acid sequence for the rodent and human mature BDNF is identical. Therefore produced mature BDNF protein is very similar in these species, and can be detected by similar methods. There are some differences with the protein folding thus the pro-BDNF form can differ between the different species (Aid et al., 2007).

Figure 2 depicts the crystalline structure of the Trk and p75 NTR receptors.

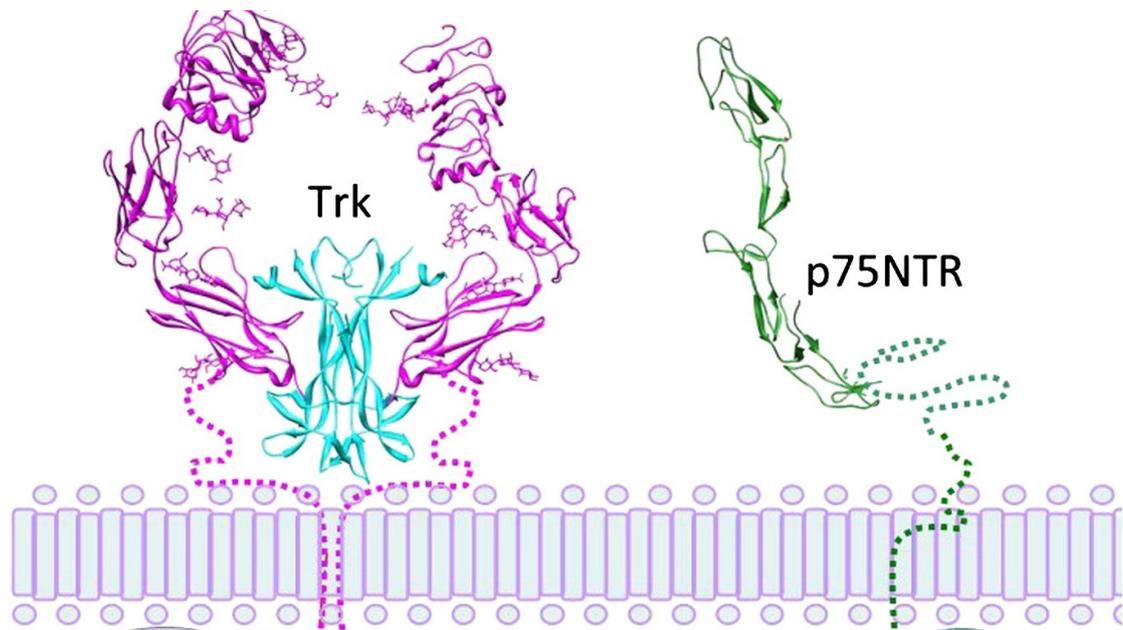


Figure 2. The crystalline structure of Trk and p75^{NTR} receptors, image edited from (Allen et al., 2013) with permission from Elsevier

1.1.1 BDNF antibody production

Antibodies are commonly produced by injecting an antigen into a target animal and allowing the animal's immune response to produce antibodies.

The antibodies used in the present study were monoclonal. The major advantage of monoclonal antibodies is that they target only one specific type of antigen. The BDNF antibodies used in the present study were produced by immunizing mice with chick, fish and mammalian BDNF (Kolbeck et al., 1999). Briefly, the mice were injected intraperitoneally with 70µg of fish BDNF in phosphate buffered saline (PBS), which were followed with three boosts of human BDNF 100µg each and four additional boosts with fish BDNF 70µg each. Three days before fusion, one mouse received three intravenous injections of human BDNF in PBS 100 µg each. Next, the animal's spleen cells were fused with non-secreting mouse myeloma cells. The positive hybridomas were cloned using a cell sorter. The positive hybridoma clones were selected in an immunoassay with immobilized neurotrophins. These monoclonal antibodies have been shown to preferentially and specifically detect BDNF protein (Kolbeck et al., 1999).

1.2 Enzyme-linked Immunosorbent Assay as Analytical Method

The ELISA method was invented by Eva Engvall and Peter Perlman in 1970. The first assay used cellulose as a particulate immunosorbent, but quickly was switched to plastic due to the tedious phases of centrifugations. The application of the antibodies to the plastic resulted in a simple, robust, inexpensive, and efficient test. The test was initially tested in primitive conditions in East Africa. After the primary launch of the method, the applications of the method have been cited in numerous publications. Engvall and Perlman received the analytical biochemistry prize from the German Society for Clinical Chemistry and the Smith Kline Bio-Science Laboratories award from the Clinical Ligand Assay Society. Engvall never patented the method. (Engvall and Perlmann, 1971)

Immunoassays are used as analytical methods to detect analytes with antibodies. Enzyme immunoassays use enzymes attached to one of the reactants in an immunoassay to allow quantification. The quantification of analytes is done through the development of colour or luminescence in addition to an enzyme specific substrate or chromogen.

Most commonly used ELISA method is the so called sandwich ELISA. The basic principle of the method is as follows: an antibody is attached to the bottom of an ELISA plate. Generally a material is used on the bottom of an ELISA plate that allows for the antibodies to attach easily, an example of a specific ELISA plate material is Maxisorb from Thermo Scientific. Next the target protein (or a biological sample containing the analyte of interest) is added which then attaches to the antibody. The secondary antibody which has been labelled with an enzyme attaches itself to the target protein, and lastly an enzyme specific substrate is added which produces a detectable reaction. If the reaction is colorimetric, the reaction can be measured by eye or a spectrometer. For quantification a standard curve is analysed along with the analytes to solve the concentration of the analyte.

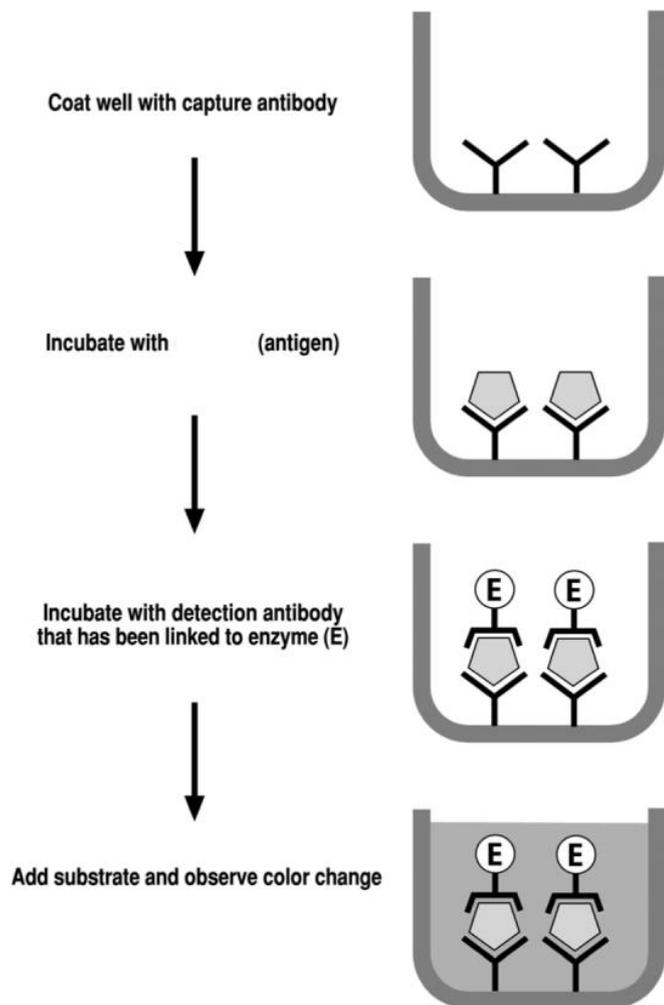


Figure 3. Sandwich ELISA from (Leng et al., 2008) with permission from Oxford journals

The advantages of sandwich ELISA are its high specificity, suitable for complex samples, flexibility and sensitivity. The high specificity comes from the two antibodies that are used thus the antigen is captured with a higher chance of it being the correct analyte than in other methods. The method's suitability for complex samples is possible due to the fact that the samples do not need any purification prior to the analysis. The flexibility of the analysis is achieved due to the many possible detection methods.

The main disadvantages of the ELISA method are the manual labour phases, the margin for error, and the time taken for the analysis to be complete. The method often involves pipetting samples individually into wells which requires quite large amounts of effort if a bigger batch of samples needs to be analysed. The margin of error increases as the amount of phases of the method increase. The possibility of human error increases every phase that there is human interference. The reliability of the materials

used also affects the error margin of the method. The accuracy should be followed with quality control. When the method is conducted correctly, the ELISA method is a valuable and affordable method in the laboratory.

2 Materials

The samples, buffers and the antibodies used in the present study are listed in detail below. Samples:

The samples in this experiment were chosen to depict the applications of the method and to determine the best combination of antibodies. The applications of the method include clinical studies of BDNF levels in the human blood and research projects using animal tissues for BDNF analysis. The standards were the same as in the previous method which would depict how well the new antibody combination would perform. Reference antibodies were used in parallel with new, tested, ones.

The following samples were chosen:

- Serum
 - (a) Human serum collected from six individuals and pooled together, anonymous sampling
 - (b) Blood extracted from adult rats. Extracted straight from heart with syringe
 - (c) Blood extracted from adult mice. Extracted with the same method as the rat samples
- Brain tissue
 - (a) Mouse brain lysates from postnatal day 1-3 old wild type, BDNF^{+/+} and BDNF^{-/-} mouse pups. Whole brain collected and frozen in -80°C until brain was lysed.
- Standard recombinant human mature BDNF
 - (a) 10 µg/ml stock BDNF diluted to standard concentration for standard curve

Reagents and materials:

The reagents and materials used were the same as the original method described in Karpova et al (2010). The reagents include the antibodies used in the experiments as well as the buffers needed for the ELISA protocol:

Buffers:

- Coating buffer
 - (a) Carbonate buffer: 50 mM NaHCO₃ to which ~210 ml of 50 mM Na₂CO₃ added until pH 9.7 is reached
- Blocking buffer
 - (a) Hanks buffer with 2% BSA and 0.1% Triton X-100
- Washing buffer
 - (a) PBS-T (1:10 dilution of 10x PBS in MilliQ water with added 0.1% Tween
- Secondary antibody dilution solution
 - (a) Hanks buffer with additional 6.66% bovine serum albumin (BSA) and 0.66% Triton X-100
- Hanks Buffer (pH 7.4)
 - (a) 125 mM NaCl
 - (b) 5 mM KCl
 - (c) 1,2 mM NaH₂PO₄
 - (d) 1 mM CaCl₂
 - (e) 1,2 mM MgCl₂
 - (f) 1 μM ZnCl₂
 - (g) 10 mM Glucose
 - (h) 25 mM HEPES
 - (i) 0,25 % BSA
 - (j) NaOH for pH adjusting if needed

The antibodies were received in 2005 and stored at -80°C before testing. The antibodies were labelled by corresponding letters to make the identification easier.

Codes and concentrations:

Primary antibodies:

Ab_{1(A)} 50 μl aliquots 1,618 μg/ml

Ab_{1(B)} 20 μl aliquots 3,60 μg/ml

Ab_{1(C)} 15 μl aliquots 5,78 μg/ml

Ab₁ reference antibody

Secondary antibodies:

Ab_{9(D)} 3,92 μg/ml

Ab_{9(E)} 16,70 μg/ml

Ab₉ reference antibody

Antibodies Ab_{1(A)}, Ab_{1(B)}, Ab_{1(C)}, Ab_{9(D)}, Ab_{9(E)}, are hybridoma clones of the reference antibodies thus are labeled with corresponding letters.

The reference antibody combination was used as a control to compare the new combinations with standard combination.

Materials:

- ELISA plate, Maxi Sorp Nunc Immunoplate (Thermo scientific)
- Multi-channel pipette (Biohit eLine pro)
- Plate mixer (Thermo scientific Mini mix)
- Plate shaker (Labsystems Well Mix)
- Plate washer (Thermo scientific Well Wash AC)
- Cold room
- Perkin Elmer Victor3 1420 Multilabel Counter

The buffers used in the study were prepared ahead of time so that there was enough buffers to last through the whole study so that the results could be compared. The ELISA plates were also kept constant as they were found to be the same lot. The plate shakers and the washer were the same throughout the study.

3 Method

The following chapter depicts the flow of the method including the sample collection and the basic BDNF ELISA protocol.

Sample Collection

The samples needed for the experiments were from live specimens, thus the preparation required separate protocols which will be described below. Samples were collected from mice, rats and humans. The human blood samples were collected from volunteers and samples were kept anonymous. The blood samples were taken by an experienced technician with appropriate training and experience for the blood collecting procedure. The animal samples were taken from animals that were under the animal license ESAVI-2010-09002/Ym-23.

3.1.1 Rodent Brain Tissue Collection and Genotyping

Mouse brain tissue was collected from wild-type, BDNF^{+/+} and BDNF^{-/-} mice. BDNF^{-/-} mice show abnormal development and typically die before postnatal week two (Ernfors et al., 1992). Therefore, the brain tissue samples were extracted from postnatal day 1-3 old mouse pups. Briefly, after sacrifice the animal was decapitated. The skin above the skull was cut from the back of the head along the mid line towards the nose. With the skull exposed the skull was cut in a similar fashion as the skin, cut along the midline the scissors moving in an upwards movement as not to destroy the soft brain tissue. The skull was removed with tweezers by bending the skull halves towards the outside. The brain was then exposed and with tweezers closed the brain is scooped out and deposited into a clean eppendorph tube and frozen with dry ice and after stored in a -80°C freezer until further processing.

Next the brains were lysed in NP lysis buffer using glass-Teflon to allow for the BDNF extraction. The buffer consists of Nodinet detergent which breaks down cell membranes and solubilizes BDNF. After the brain is completely lysed the solution is centrifuged at 16 000 g in 4°C for 10 minutes. The resulting supernatant is collected into a clean eppendorph tube and frozen for the analysis. (Karpova et al., 2010, Karpova, 2013)

The animals that were used for the samples needed to be genotyped so that the samples could be identified either as knock-out, heterozygous, or wild-type tissue. The genotyping samples were collected at the same time as the brain tissue samples. A tail piece of the specimen was collected and transferred into a clean eppendorph tube. The tailpieces were digested with a commercial mouse genotyping kit (KAPA mouse genotyping kit). PCR was performed for the digested DNA samples. After the PCR the resulting DNA fragments were separated with gel electrophoresis. The gels were treated with ethidium bromide so that the DNA segments would be clearly visible under ultra violet light. After the samples had run into the gel for a time period of ~20 minutes, the gel was imaged with UV light. The results were interpreted from the image. The DNA fragments are sized as following: wild type fragment ~275 base pairs, heterozygous fragments ~275 and ~340 base pairs, knock out fragment ~340 base pairs. The following image depicts the fragments by which the genotyping result is decided.

Figure 4 depicts the genotyping results of BDNF-null mice brain tissue:

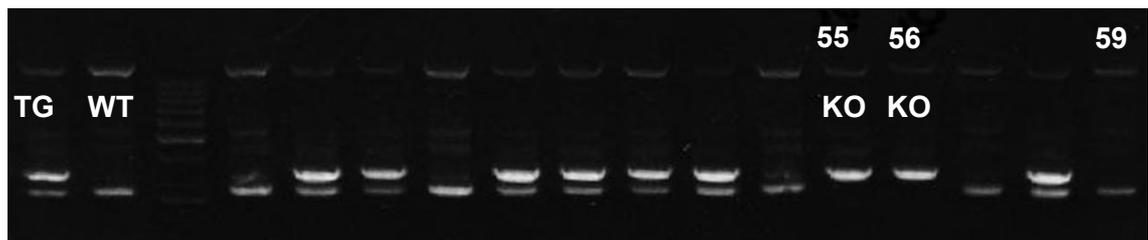


Figure 4. BDNF Genotyping image. From left Heterozygous control and wild type control and 50 bp marker. Samples 46-59. 55 and 56 are knock-out mice

The genotyping ensures that the samples are the correct genotype so that they can be used as controls for the analysis. The method requires testing so the genotyped brain samples are used to test if the method can detect different amounts of BDNF from rodent brain tissue.

3.1.2 Acidic Treatment of Brain Tissues

After the brain tissues were lysed the samples had to be acid treated to free the possible BDNF from the binding proteins usually associated with BDNF. The binding proteins generally inhibit the immunological recognition of the BDNF thus resulting in an unreliable amount of BDNF in the sample (Okragly and Haak-Frendscho, 1997). Briefly, 20 μ l of sample lysate was diluted into 175 μ l of Hanks buffer which was acidified with 6 μ l of 1 M hydrochloric acid (HCl) and allowed to incubate at room temperature for 15 minutes. The pH of the sample was measured with broad range (1-14 pH) pH paper. The pH of the solution had to drop to pH 3 during incubation. After the incubation period, 6 μ l of 1 M sodium hydroxide (NaOH) was added to neutralize the solution. The pH of the solution was measured again to detect the neutralization of the solution. The pH of the solution after neutralization had to be pH 7 so that the ELISA analysis could be conducted (Okragly and Haak-Frendscho, 1997).

3.1.3 Blood Collection from Rats and Mice

Blood samples were collected from adult rats and mice. Briefly, the animal was stunned with CO₂ and the neck was dislocated.

The animal was then turned so that it was lying on its back and a needle was inserted gently to the right sternum. The needle was inserted approximately one centimetre into the thorax and then the sample of blood was extracted straight from the heart. From a rat approximately 10 ml of blood was collected and from a single mouse approximately 3 ml of blood was collected. The samples were allowed to incubate at room temperature for approximately one hour so that the blood coagulated. After the hour the samples were spun down at 3500 rpm for 15 minutes. After the samples were spun down the supernatant was decanted into clean tubes and frozen until the serum samples were needed for analysis.

3.1.4 Human Blood Sample Extraction

Six adult voluntary individuals were chosen (three female and three male) and blood was collected intravenously with a vacuum tube. The samples were collected in the same fashion as in clinical trial of BDNF. Generally the blood samples are collected without vacuum as not to activate the BDNF found in blood platelets. So that the results of the analysis could be compared to the clinical trial method the samples were collected with a vacuum tube. Samples were labelled with numbers randomly so that the anonymity was preserved. Samples were allowed to stand for an hour at room temperature and then centrifuged at 3500 rpm for 15 minutes. The serum was removed into clean tubes, pooled and frozen until the samples were to be used in the analysis. Samples were collected by Outi Nikkilä, Research Technician.

3.2 Basic ELISA Protocol

The following flow chart in Figure X illustrates the progression of the ELISA protocol:

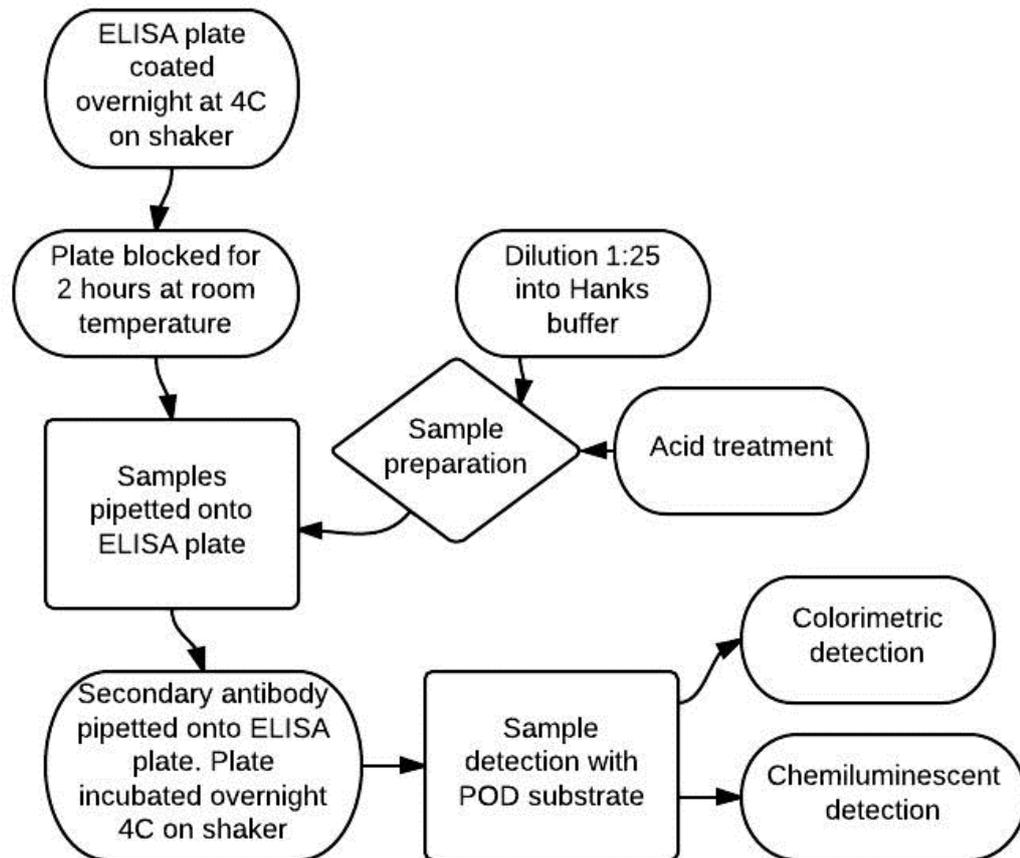


Figure 5. Flow chart of the ELISA protocol

To familiarize the method, the ELISA was done essentially as described in Karpova et al (2010) using the reference antibodies. Briefly, the preparation of the 96-well plate was the first step of the method. The preferred material of the 96-well plate was Maxisorb® (Thermo Scientific). The primary antibody was diluted to suitable concentrations (1:4000). Pipetted 200 µl/well of antibody dilution and sealed the plate. Plate was incubated overnight at 4°C in the cold room on a shaker at level 4 of the shaker (approximately 400 rpm) to ensure proper attachment of the antibody to the bottom of the well. The next day the coating buffer was removed and the plate was tapped onto a paper towel to remove excess coating buffer. Next the plate was blocked with 300 µl per well of blocking buffer for two hours at room temperature on a shaker 400 rpm.

Samples were prepared with an acid treatment if needed. For human, rat, and mouse serum or plasma acid treatment was not required. Samples were diluted 1:25 into Hanks buffer. Also the standard curve dilutions were prepared (1000, 500, 250, 125, 62.5, 31.2 pg/ μ l) 170 μ l of the sample dilutions was added to each corresponding well. 30 μ l of peroxidase conjugated BDNF antibody dilution is added to each well. The plate was left to incubate over night at 4°C on a shaker at 400 rpm. Figure 5 depicts a chart used for the analysis.

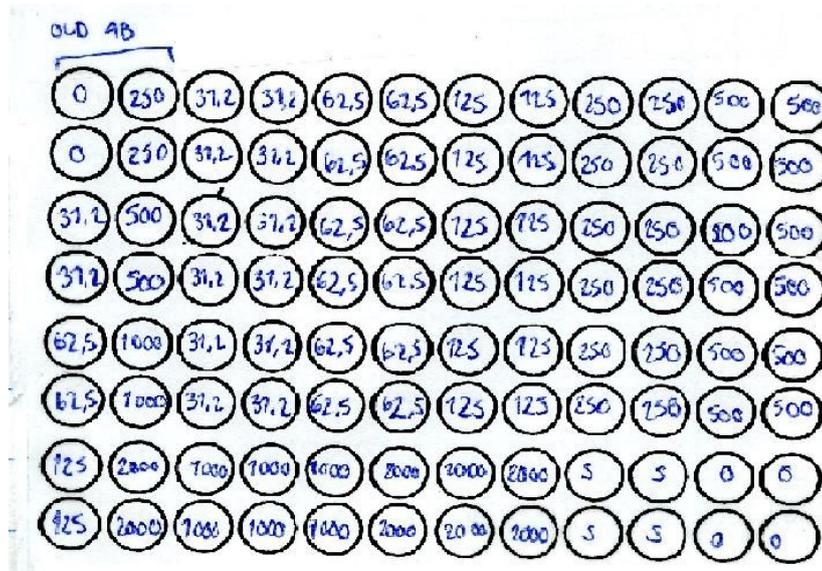


Figure 6. Pipetting chart for BDNF ELISA

On the final day the plates were washed four times with 300 μ l/well of PBS-T. After washing, 200 μ l of POD substrate was added to each well and allowed to incubate for 20 min in room temperature in a dark place. The reaction was then stopped with 50 μ l of sulphuric acid (1N H₂SO₄). The intensity of the colour was measured with the spectrometer Victor at the wave length 490 nm (Karpova et al., 2010).

3.3 Antibody Combinations

The new possible antibody pairs were tested separately so that the best pair could be determined.

Firstly the protein concentration of antibodies measured with spectrometry at wave-length A280 nm (Biorad). After protein concentration analysis proper dilutions of antibodies were made so that they were equal to the concentration of the previous anti-

body combination (Ab_1 and Ab_9). The concentrations of the antibodies were also changed to determine the best combination. The concentrations of the antibodies were increased three fold from the previous concentration and also decreased three fold from the 'old' concentration.

3.4 Detection Methods

The original detection method was a colorimetric detection, the reagent produced a colored reaction when exposed to the POD labelled secondary antibody. The POD reagent used for the method was a chromogenic substrate for the peroxidase-mediated colour development in ELISA assays. The POD Blue substrate turned a dark blue colour when in contact with the POD conjugated antibody and after the stop reaction liquid was added the colour changes from the blue to a bright yellow hue. The absorbance was read at 490 nm on a spectrometer (Roche, 2007). The more antibodies attached to the plate the higher the signal from the color forming reagent. The overdevelopment of the color was a concern with the method as the substrate began to precipitate as the stop reaction was added if the plate had been incubated for an unsuitably long time.

Also a chemiluminescence was tested to improve the sensitivity of the method and ease the last steps of the analysis. Chemiluminescence is based on the signal generated in enzyme-catalysed light emitting reactions which is similar to the signal generated during radioactive decay. The horseradish peroxidase (POD) in the presence of hydrogen peroxide (H_2O_2) catalyses the oxidation of diacylhydrazides like luminol. A reaction product in an excited state is formed, which then decays to the ground state by emitting light (Roche, 2010). The light emitted can be measured with a chemiluminescence reader. The chemiluminescence reagent does not require a stop reaction after the incubation period so one phase of the analysis could be left out, lessening the human error aspect as the mixing phase of the stop reaction can be conducted in many ways differing from person to person. The chemiluminescence detection method requires an opaque white plate so that the signal can be detected.

Figure 7 depicts the reaction that produces the light signal in the chemiluminescence method:

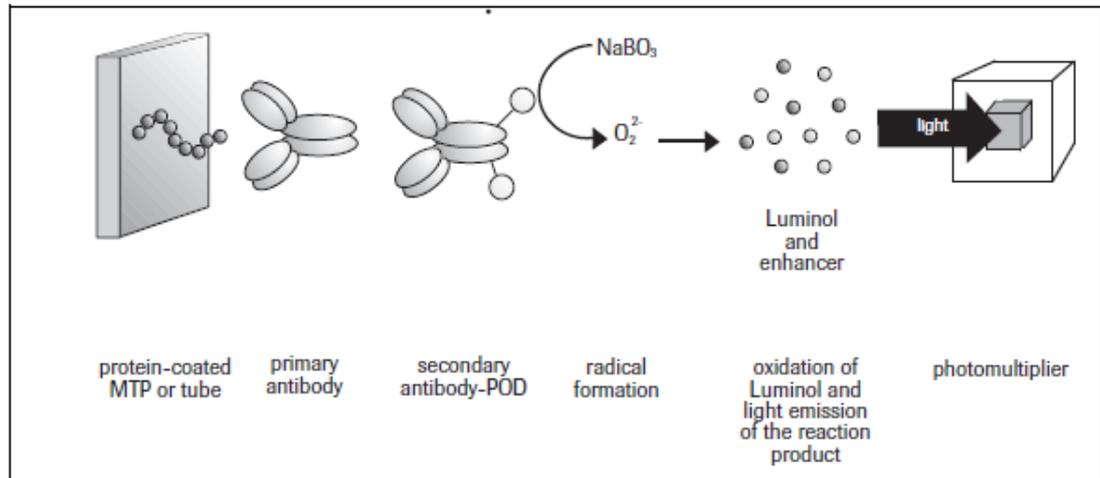


Figure 7. Enzyme-induced generation of chemiluminescence and detection by photomultiplier. (Roche, 2010)

3.5 Data Analysis

The following variables were analysed in the basic protocol:

- Sensitivity – How well the analysis determines the presence of different BDNF molecules.
- Selectivity – Does the method detect other neurotrophins. Origin of the molecule can be from mice, humans or rats.
- Standard curve – The standards form the standard curve from which the quantity of BDNF can be calculated, these calculations include the dilution factor of the samples.

The variables that are analysed allow for the conclusion to be made which of the antibody combinations are the most suitable for the sample range that are to be analyzed after the optimization is completed for the method.

4 Results

The main findings of the experiments are outlined per experiment in the following sections: The following flow chat illustrates the proccession of the whole thesis experiments Figure 8

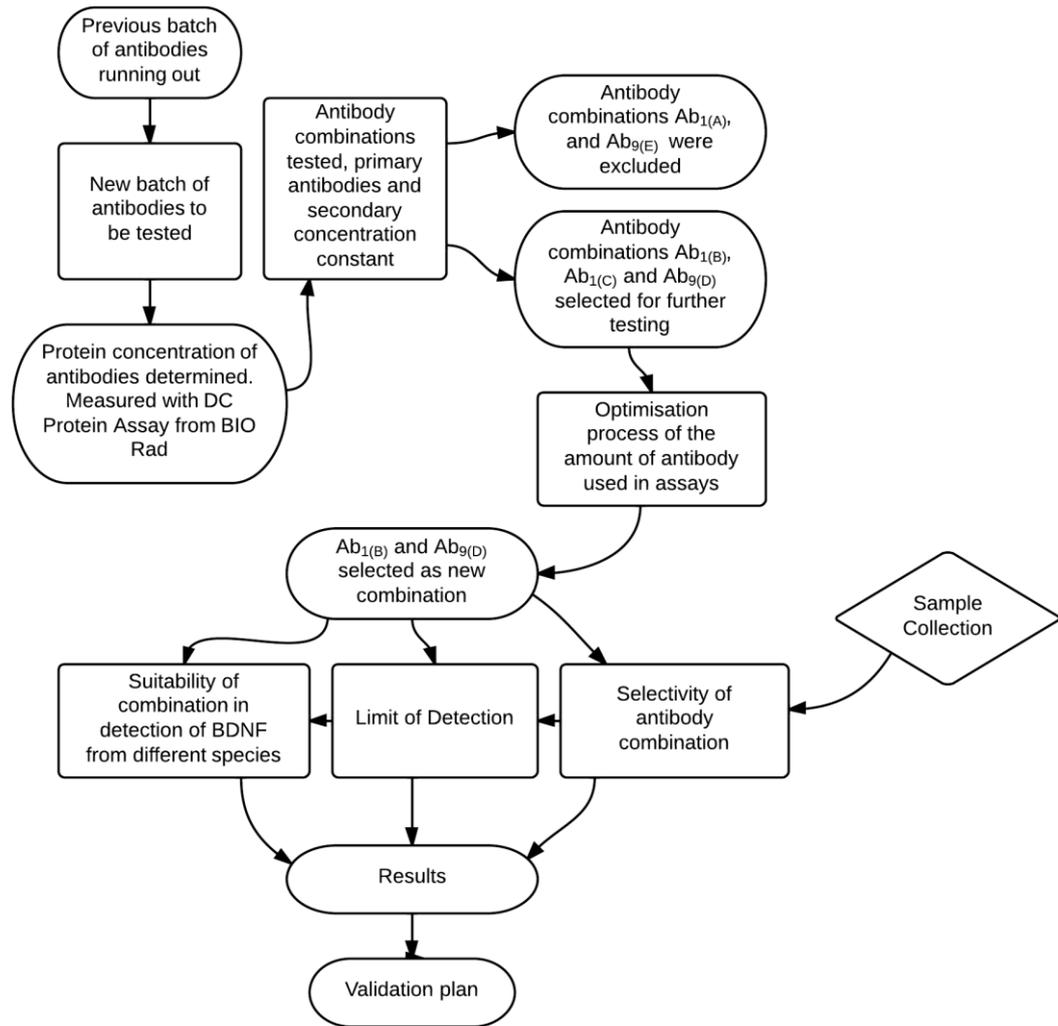


Figure 8. Flow chart of the progression of the method

Experiment 1: Antibody combinations

All the primary and secondary antibodies were arranged into combinations so that all possible combinations were tested. In total six combinations of primary and secondary antibodies were tested. The antibody concentrations were also tested so that the concentrations of the antibodies were the same as the old antibody combination, three fold less, and three fold more than the original concentrations. Figure 9 illustrates the antibody combinations that would be tested in the experiments

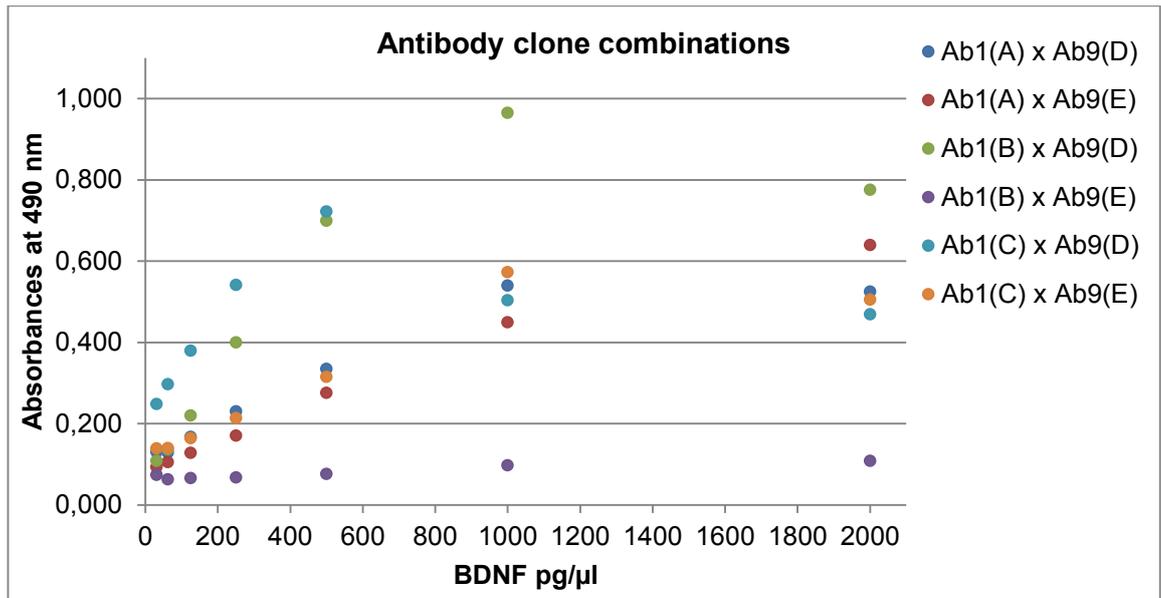


Figure 9. : The Antibody combination detection of BDNF standard.

The antibody combinations that were excluded after the first combinations were tested were: $Ab_{1(A)} \times Ab_{9(D)}$, $Ab_{1(A)} \times Ab_{9(E)}$, $Ab_{1(B)} \times Ab_{9(E)}$, $Ab_{1(C)} \times Ab_{9(E)}$. The antibody combinations that were the most linear were chosen for further testing. The combinations chosen as promising combinations for further testing were $Ab_{1(B)} \times Ab_{9(D)}$ and $Ab_{1(C)} \times Ab_{9(D)}$. The first experiment was to test the amount of primary antibody concentrations. The secondary antibody was kept constant, while the primary antibody concentrations were changed. Figure 4 illustrates the antibody combinations that showed interesting results that were to be tested further:

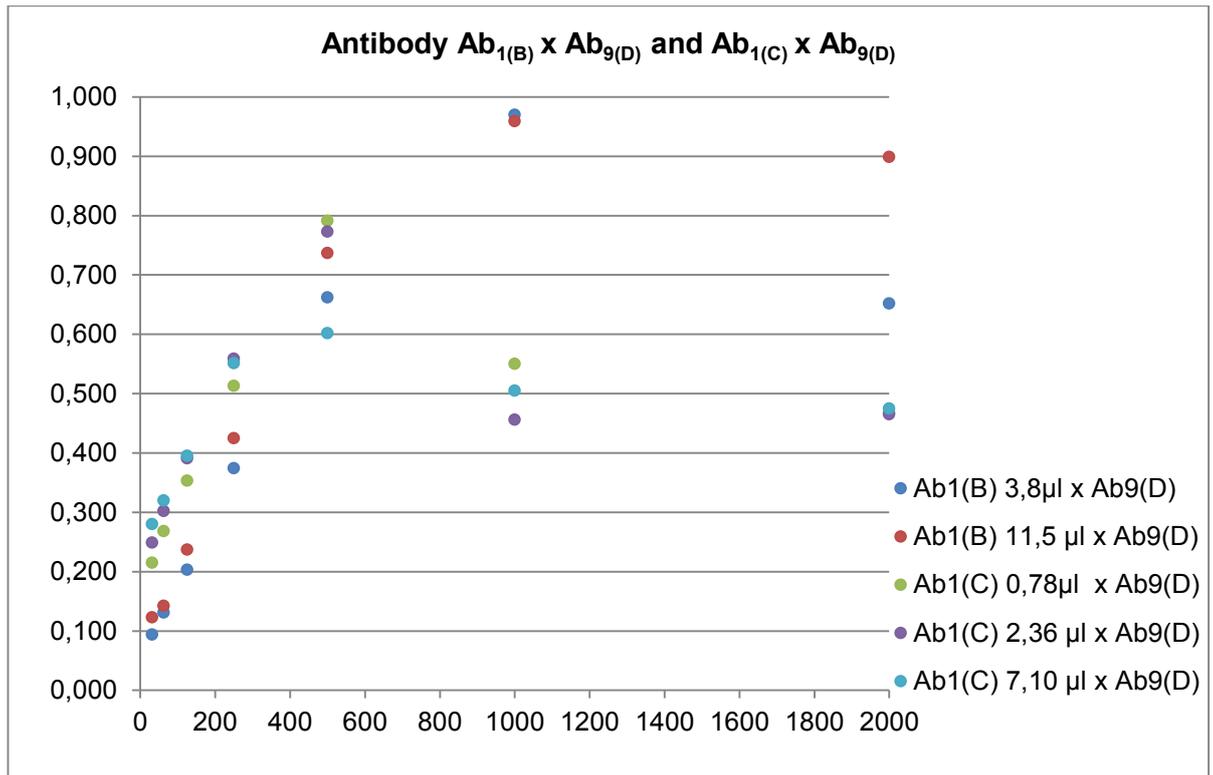


Figure 10. Antibody $Ab_{1(B)} \times Ab_{9(D)}$ and $Ab_{1(C)} \times Ab_{9(D)}$ combinations. The primary antibody concentration was changed with each combination. The volume of primary antibody pipetted into the wells was as following: $Ab_{1(B)}$ 3,8 µl and 11,5 µl and for $Ab_{1(C)}$ 0,78 µl, 2,36 µl and 7,10 µl

The standard dilutions were limited up to 1000 pg/µl due to the nature at which the samples began to persipitate as the concentration of the sample went above 1000 pg/µl. The persipitaton in the color reaction caused the absorbance to taper significantly thus causing issues with the linearity of the experiments. The future tests were completed with the standard curve dilutions ranging from 31,2 pg/µl to 1000 pg/µl.

The antibodies were diluted to the same concentration as the old antibodies. The pipetted amounts of antibody dilutions took into account the old antibody concentration and from that the three fold raise or decline of concentration. The $Ab_{1(B)}$ amounts of antibody that were to be tested further were 3,8 µl, 11,5 µl. The threefold increase of the concentration was not taken into account as the linearity was unacceptable to be used as a combination to be tested further. A possible error in the method for the combination occurred. For $Ab_{1(C)}$ the amounts of antibody that were to be tested were: 2,3 µl, 7,1 µl and 21,3 µl.

After the first test of the first experiment, the selected combinations were tested with different concentrations of secondary antibody. The amount of antibody was changed in the same method as in the first test of the experiment 1. The secondary antibody concentration was kept at the same concentration as the reference antibody, increased by three fold decreased by three fold. The amount of secondary antibody pipetted onto the plates was as following: 9 μ l, 3 μ l, and 1 μ l.

Figure 11 depicts the standard curve for the antibody combinations $Ab_{1(B)}$ 11.5 μ l and $Ab_{9(D)}$ 1 μ l, 3 μ l and 9 μ l, where as Figure 12 depicts the antibody combination chosen for futher thesting. :

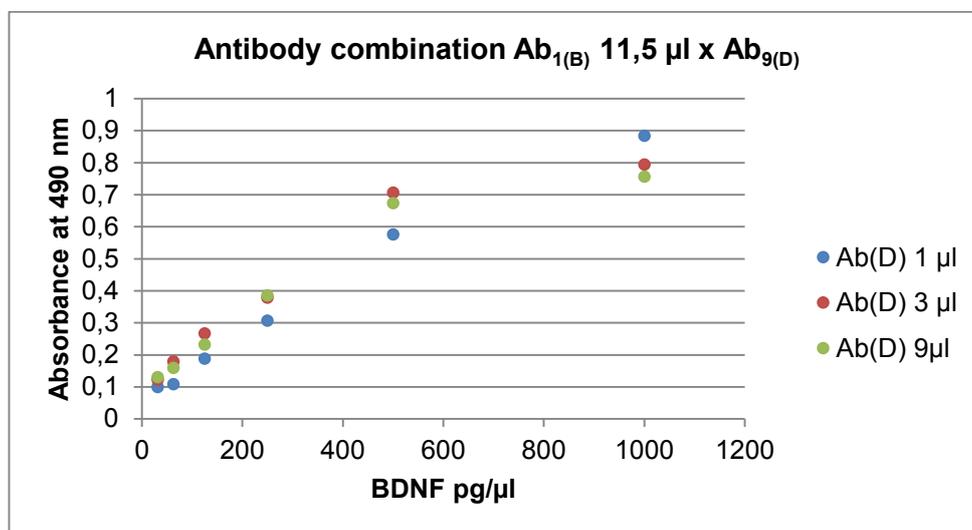


Figure 11. Antibody combination $Ab_{1(B)}$ and $Ab_{9(D)}$

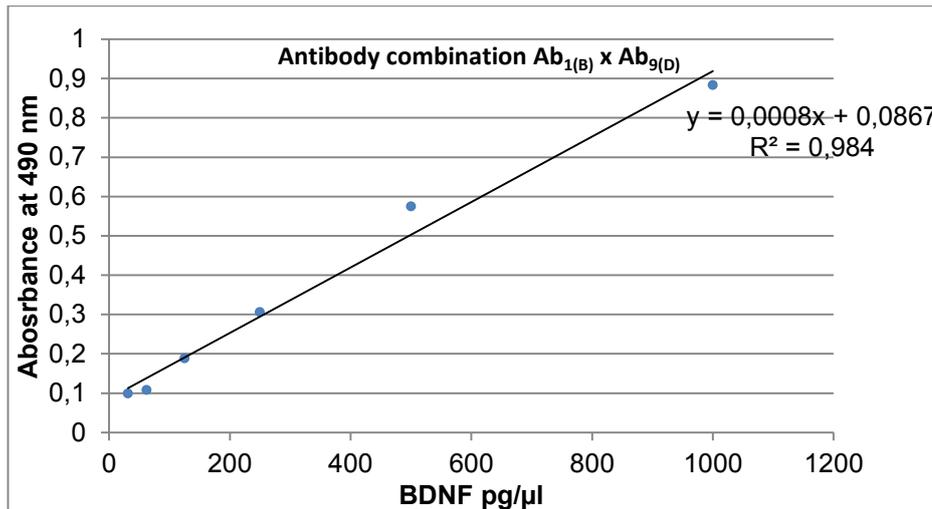


Figure 12. Antibody combination $Ab_{1(B)} \times Ab_{9(D)}$. The volumes pipetted 11,5 μl $Ab_{1(B)}$ and 3 μl for $Ab_{9(D)}$. The combination gave the most linear curve thus being the most promising antibody combination.

The most promising antibody combination was found to be $Ab_{1(B)}$ 11.5 μl , $Ab_{9(D)}$ 3 μl . The combination was used for further testing.

Experiment 2: Methods of Detection

The antibody combination $Ab_{1(B)} \times Ab_{9(D)}$ was chosen to determine the sensitivity of the method. The standard curve concentrations were diluted up to a known 7.8 $\text{pg}/\mu\text{l}$ and the maximum was 1000 $\text{pg}/\mu\text{l}$. The analysis was able to detect a signal from the concentration 15.6 $\text{pg}/\mu\text{l}$ upwards until 1000 $\text{pg}/\mu\text{l}$ with the colorimetric method. The absorbances were above the limit of detection, the absorbance values were ranging from 0.032 to 1,039. The analysis gave a signal for the concentration 7.8 $\text{pg}/\mu\text{l}$ as well but the signal was very weak and could not be included as a detectable concentration. Two methods of detection were used for the analysis, chemiluminence and colorimetric reagents.

Figures 13 and 14 illustrate the signal intensity of the concentrations:

Calorimetric results:

The graph illustrates the absorbance of the BDNF standards after background subtraction. The absorbances below 15,6 $\text{pg}/\mu\text{l}$ are below the detectable level of the analysis.

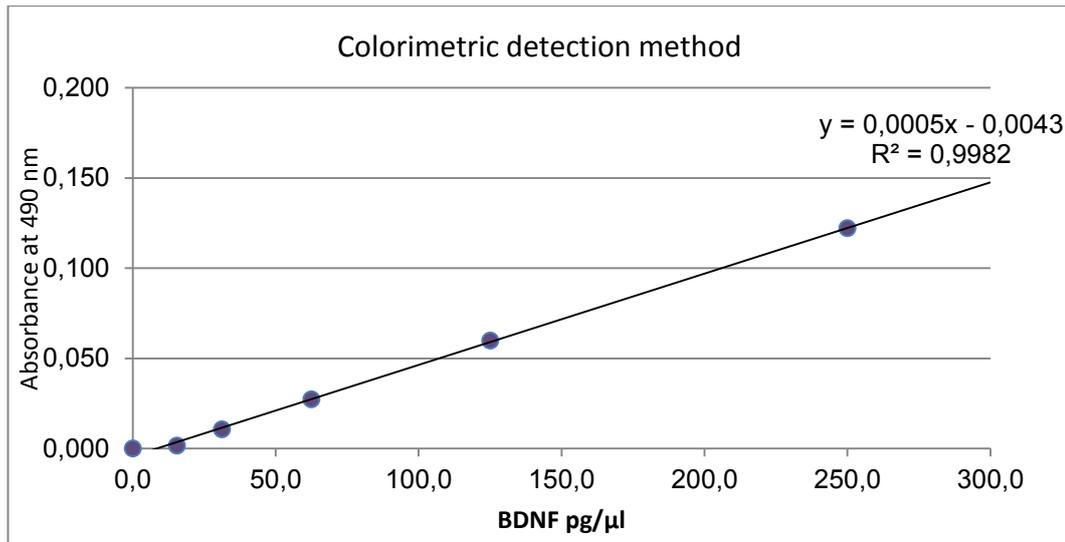


Figure 13. Standard curve of the colorimetric detection method for the antibody combination Ab1_(B) 11,5μl X Ab9_(D) 3μl

Chemiluminescence results:

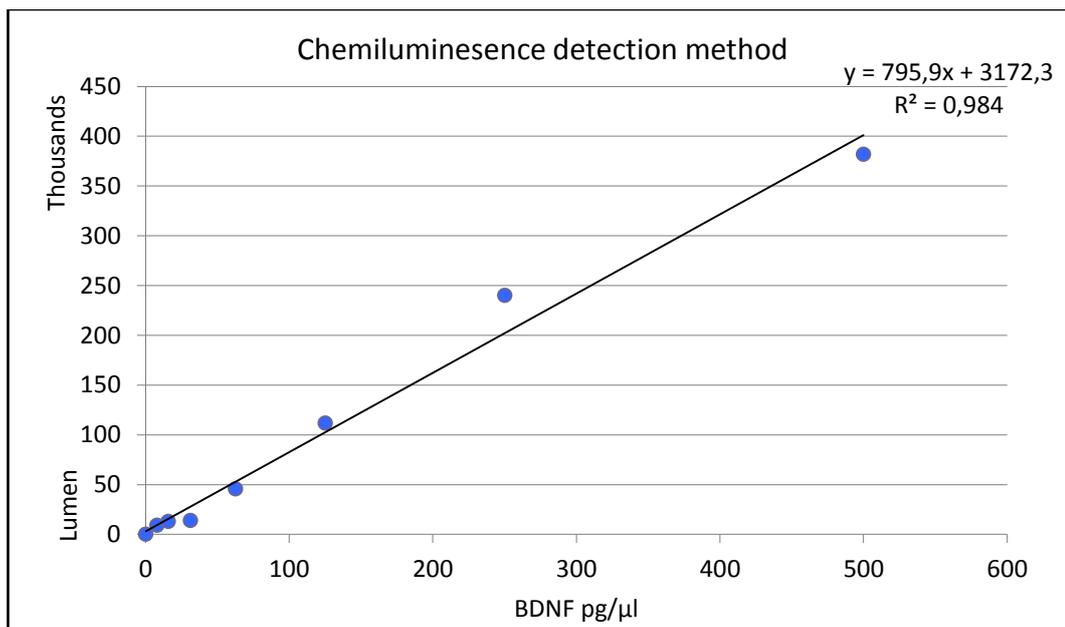


Figure 14. Standard curve of chemiluminescence detection method for the antibody combination Ab1_(B) 11,5μl X Ab9_(D) 3μl

The chemiluminescence method gave a clear signal for 7,8 pg/μl of BDNF after background subtraction, which suggests that the chemiluminescence method would have a lower detection limit. The method though required more optimisation. The method of

detection could be changed to favour the chemiluminescent method due to its higher sensitivity.

Experiment 3: Sensitivity and Selectivity

As the antibody combination was established and the limit of detection detected, the next step was to determine the sensitivity and selectivity of the combination. The selectivity was measured by how well the antibody combination recognized BDNF from different species including human, rat, and mouse serum samples and whether or not the antibody combination recognised other neurotrophins from the same family.

Figure 9 illustrates the ability of the antibody combination to detect the amounts of BDNF from a mouse strain of Knock out, Heterozygous, and wild type animals. The graph illustrates the results of the wild type expressing the most BDNF while the heterozygous expressing less and the knock out expressing an undetectable quantity of BDNF (Barde et al., 1982).

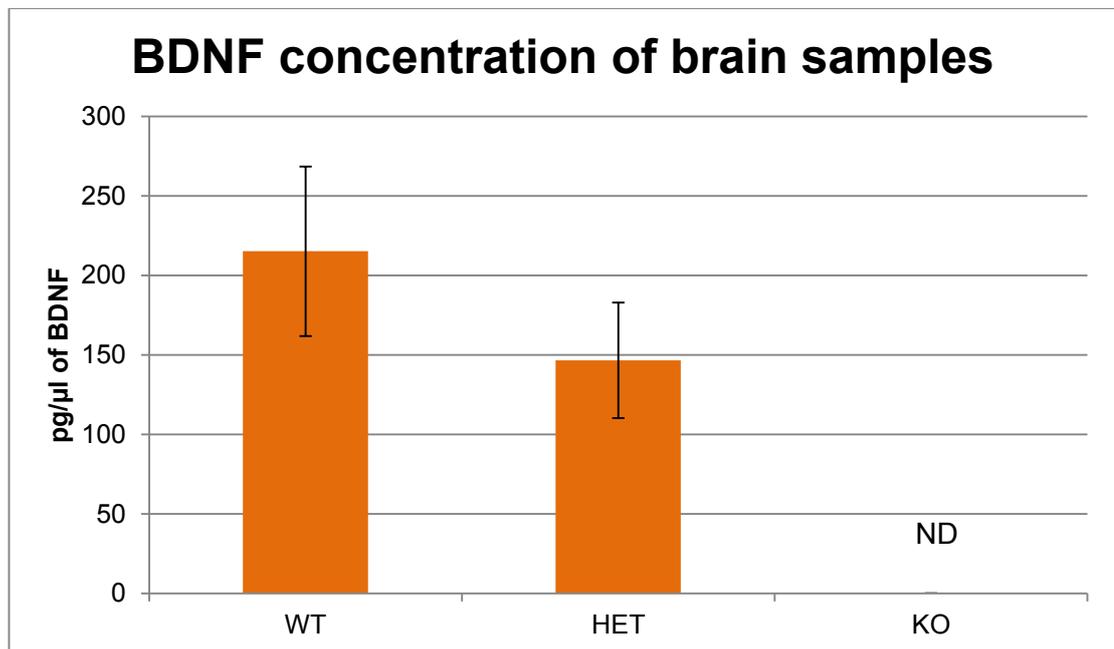


Figure 15. BDNF concentration detected from Bdnf wild type (wt), heterozygous (het) and knock-out (ko) tissues. Knock-out tissue samples were not detected (ND). Samples concentration calculated with a standard curve: $y = 0,0007x + 0,0004$. Samples had a confidence interval of 95%, for Wild type tissue 53,34, and for Heterozygous 36,35.

The figure 15 depicts the different amounts of BDNF detected from BDNF-null brain tissue lysates. The antibody combination produced a signal for the human, rat and mouse plasma and human and rat plasma samples are shown in Table 1.

		Abs	pg/ μ l
Human	Serum	0,743	642,2
	Plasma	0,119	18,2
Rat	Serum	0,121	20,2
	Plasma	0,137	36,2

Table 1. Human and Rat blood samples. Concentration calculated from a standard curve $y = 0,0062x + 0,0188$ with known concentrations ranging from 31,2 to 1000 pg/ μ l.

The absorbances of the plasma and serum samples was above the detection limit and gave probable concentrations for the samples.

. The following figure 16 depicts the detection of the other neurotrophins of the same family along side BDNF.

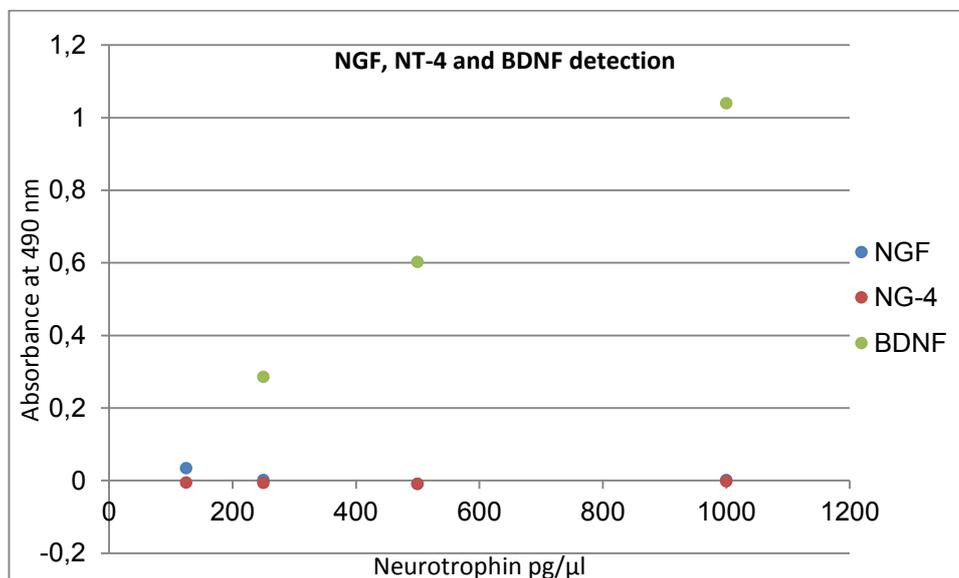


Figure 16. Other neurotrophin detection from the same family of neurotrophins as BDNF

The antibody combination was not able to detect the other neurotrophins that were tested. NGF and NG-4/5 were not detected though they are from the same family as BDNF and were at a high concentration of 1000 pg/μl. The absorbances were under the detection limit thus neither of the neurotrophins was detected by the antibody combination. The results clearly depict the thought process through which the antibody combination was chosen and deemed to be reliable for the new method.

5 Discussion

In this study it was observed that a new antibody combination was found and was set up so that the method could be used in the laboratory to quantify the amount of BDNF in tissue, blood and cell culture samples. The antibody combination was more sensitive than the combination that had been previously been used. Validation would be needed in determining how reliable the method would be in the long run. A suggestion for this would be an inner control of the pooled serum samples.

5.1 Samples

The data points that the changing the antibody concentrations improved the sensitivity and selectivity of the method. The concentration changes were as following, keeping the secondary antibody concentration constant to our reference secondary antibody and keeping the concentration of the primary antibody constant to our reference primary antibody. Due to the antibody concentration differentiation between the reference antibodies and the reference antibodies, $Ab_{1(B) 11,5\mu l}$ is at the same concentration as Ab_1 concentration. The observed increase in sensitivity and selectivity was found with the antibody combination $Ab_{1(B)11,5\mu l}$ and $Ab_{9(D) 3 \mu l}$. The amount of antibody pipetted into the buffers would be changed to 11.5 μl of the $Ab_{1(B)}$ into 26 μl of carbonate buffer and 3 μl of $Ab_{9(D)}$ into 3.8 μl of coating buffer.

5.2 Sensitivity

Facing the issue with the method of not being able to detect BDNF concentrations under 31,2 pg/μl, it was tried to increase the sensitivity by changing the antibody combinations to different clones from the said reference antibodies (Ab_1 and Ab_9). The data

points to the limit of detection being 15.8 pg/ml for the POD labelled antibodies and for the chemiluminescent reagent the results are inconclusive and require optimization. The sensitivity of the standard protocol is 31,25 pg/ml so the new antibody combination improves the sensitivity significantly.

The method still needs to be optimized and requires validation in determining how well the limit of detection will stay stable. The results suggest that there is slight differation between plates which could limit the stability of the limit of detection.

5.3 Selectivity

The data points that the new antibody combination does not detect other neurotrophins of the same family, such as NGF and NT-4. NT-3 was not analyzed so we are able to conclude that with almost complete certainty that the antibody combination does not detect any other neurotrophins other than BDNF. The antibodies also did not detect BDNF from *bdnf*^{-/-} knock-out mice, but did detect intermediate levels in heterozygous animals and the most BDNF in wild type animals. The antibody combination detected BDNF in human and rat blood samples, thus suggesting that the method would be suitable for both human and rodent BDNF analysis.

5.4 Validation Plan

The method is set up so that the method has been optimized for the colorimetric method. The method though was not validated. The application of this method does not generally need comparable results as the results are being used for research purposes and not for diagnostics.

The method could be validated as a future project to determine the reliability of the method. A good method of determining how well the method behaves on the long run is an inner standard. An inner standard in this method would be a sample that is pooled and has always the same so the concentrations of BDNF. The sample should have very little variation so that the variation between sample concentrations could be followed to illustrate how the method stays stable or unstable. The concentrations of the inner standard could be followed for a long period of time to determine the standard

deviation and error margin of the method. The inner standard that was chosen for the method was a pooled serum sample of human blood from six individuals. The addition of the inner standard could possibly determine how well the method behaves on the long run.

After the standard deviation and error margins are determined the analysis could be tracked on how reliable it is by using a simple graphing program that would illustrate how well the analysis stays within the acceptable error margins. In the graph the error margins would include a 'warning' phase where the results should be scrutinized but no action would be taken to correct the result. The warning phase would be the average \pm two standard deviations. The action phase would also be included that would mean if a result would go into the phase; something would have to be done. The action phase would be the average of the results \pm 3 standard deviations.

Figure 17 illustrates the type of graphing method that could be used to determine the reliability of the ELISA method:

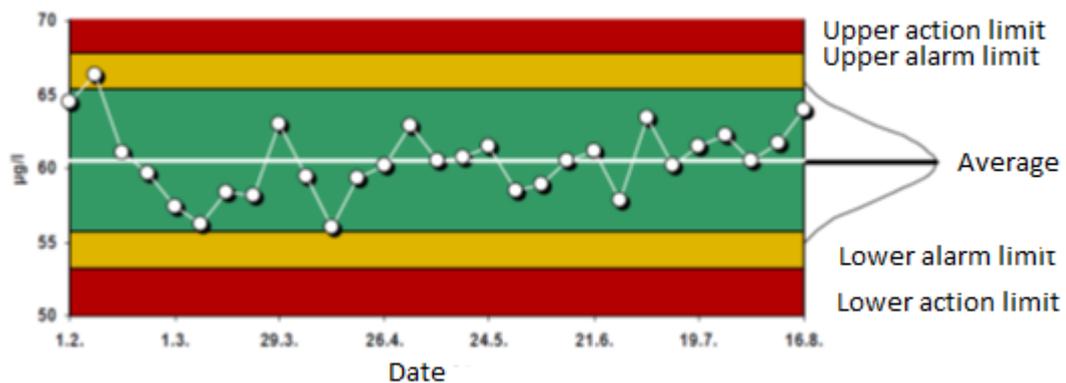


Figure 17. Picture depicting an X-chart. Image edited from (Jestoi, 2012) (Labels translated into english from finnish)

The BDNF ELISA method is known to be slightly unstable and the variation with results can vary depending on many factors. The introduction of a method of following the results could give a clear indication on the reliability of the method on a long run.

6 Conclusion

The aim of the thesis was achieved in determining the new antibody combination for the BDNF ELISA method. The antibody combination that was chosen was Ab_{1(B)} and Ab_{9(D)}. These new clones had a slightly better sensitivity than the previous combination but the sensitivity of the literature was not achieved. Due to the nature of the cloning process there can be small changes in the detection sensitivity.

The method was specific to BDNF which was a key factor in its reliability. The data shows that the antibodies do detect BDNF molecules while they do not detect other neurotrophins of the same family. The knowledge that the method is specific to the detection of BDNF is key in the reliability of the method.

The detection methods are a field in which there are other possible methods to improve sensitivity. The colorimetric method has been used in the past with success but it is not very sensitive and needs an extra step to end the colour forming reaction. The extra step of the detection process can cause unreliable data. The chemiluminescence method showed promise in improving the sensitivity of the method. The detection did not need a stop reaction which would take out the last step of the protocol which could reduce the probable error of the method. The luminescence stays constant for an hour after the reagents are added which provided a more stress-free detection. Due to time restrictions, the chemiluminescence detection method was not optimized but could be a project that could be done as another project.

A validation plan was drafted for the improved method with inner standards of human serum samples so that the reliability and transparency of the method could have followed. The validation plan could improve the criticism of the reliability of the method.

The improved BDNF ELISA method has been used regularly after the optimization. The inner standards have been used to track the reliability of the method. The method has shown to be reliable for the detection of BDNF. The aims were achieved for the thesis.

Acknowledgements

I would firstly like to express my deepest gratitude to Dr. Eero Castrén for allowing me to complete my work placement and thesis project at the Neuroscience center at the University of Helsinki in his research group. Secondly I would like to express my gratitude to Dr. Tomi Rantamäki for the total support and willingness to aid my journey through this thesis. Also I would like to express my gratitude to Outi Nikkilä for the great technical help and ever lasting patience. I would like to offer my thanks to Juha Knuutila for the aid with structuring my thesis and introducing me to the world of neuroscience. I would also like to offer my thanks to Jonita Martelius and Aira Korkeamäki for their proofreading my thesis. I would like to thank Marius Hoener and Lothar Lindemann from Roche Diagnostics for the antibodies that were the key to this project. And lastly I would like to acknowledge the everlasting support from my mother.

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BDNF Protocol BDNF ELISA

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 Date: 2.9.2013
 Description: ELISA-based method for the measurement of BDNF from mouse/rat brain and serum.
 Reliable sensitivity: ~25-30 pg/ml (Abs after background subtraction >0.05)
 Important: This protocol is based on Roche's antibodies. If scientific data obtained by this method are considered for publication, the permission should be obtained from Roche.

I. Preparation of the 96-well plate (Maxisorb®, Nunc)

- Primary BDNF antibody Ab#1_B (stored at -80°C) in 26 ml Carbonate buffer (1:4000, (e.g. 11,5 µl in 26ml; 200 µl/well).
- Seal the plate and incubate overnight at +4°C

I. Blocking

- add 300 µl/well of blocking buffer
- 2 hours at room temperature

II. Sample preparations

- Acidic treatment ("epitope release") in regular 96-well plate
 (- optimal acidic treatment conditions should be done for the selected samples)
 - For NP++ lysed brain samples (conc. ~ 3-10 µg/µl) the following conditions can be used
 - Visual cortex: 30 µl of sample + 165 µl of Hanks + 6 µl 1M HCl → 15 min → 6 µl 1M NaOH
 - Prefrontal cortex: 15 µl of sample + 180 µl of Hanks + 6 µl 1M HCl → 15 min → 6 µl 1M NaOH
 - Hippocampus: 15 µl of sample + 180 µl of Hanks + 6 µl 1M HCl → 15 min → 6 µl 1M NaOH
 - Amygdala: 15 µl of sample + 180 µl of Hanks + 6 µl 1M HCl → 15 min → 6 µl 1M NaOH
 - Caudate putamen: 20 µl of sample + 175 µl of Hanks + 6 µl 1M HCl → 15 min → 6 µl 1M NaOH
 - Nucleus accumbens: 20 µl of sample + 175 µl of Hanks + 6 µl 1M HCl → 15 min → 6 µl 1M NaOH
 - Substantia nigra: 20 µl of sample + 175 µl of Hanks + 6 µl 1M HCl → 15 min → 6 µl 1M NaOH
 - Ventral tegmental area: 30 µl of sample + 165 µl of Hanks + 6 µl 1M HCl → 15 min → 6 µl 1M NaOH
 - For NP++ lysed cell culture lysates (conc. ~1-3 µg/µl)
 - Primary CX/HC: 20 µl of sample + 175 µl of Hanks + 6 µl 1M HCl → 15 min → 6 µl 1M NaOH
 - MG87: 20 µl of sample + 175 µl of Hanks + 6 µl 1M HCl → 15 min → 6 µl 1M NaOH
- Important note: Before running lots of samples, check (randomly 1 or 2 samples) the pH value using pH stick after HCl and NaOH (HCl → pH ~3 → NaOH → pH ~7)

Without acidic treatment

- (human)/rat serum/plasma (e.g. 1:25 in Hanks)

III. Sample incubation

- 170 µl/well of sample (in Hanks buffer)
- 170 µl/well duplicates of BDNF- standards (Peprotech) in Hanks buffer (no acidic treatment)
 - Stock: 10 ng/µl aliquots (-80°C)
 - Make (1000), 500, 250, 125, 62.5, 31.2 pg/ml dilutions
- 30 µl/well of POD-conjugated BDNF Ab#9_D antibody (-80°C; 1:1800 in Hanks buffer +6.66% BSA and 0.66% Triton X-100; e.g. 3 µl in 3.8ml)
- Seal the plate and incubate overnight at +4°C

IV. Add substrate

- Wash 4x with 300 µl PBS-T
- Add 200 µl/well of BM blue POD substrate (100 ml bottle, store in dark at 4°C; Roche, take to RT 10-15 min use!).
 - Incubate for 20 minutes at RT in a dark place
 - Stop the reaction with 50 µl of 1 M H₂SO₄ (mix while pipetting)

V. Count on Victor reader

- Measure in ELISA reader at 490 nm

REAGENTS

Carbonate buffer

- 50 mM NaHCO₃ (4.2 g/l, pH 8.2; RT, reagent room) + 50 mM Na₂CO₃, (5.3 g/l, pH 11.2; RT, reagent room), add until pH 9.7 (~210 ml)
- Store in RT up to 3 months

PBS-T

- 1/10 10x PBS in MilliQ water + 0.1% Tween®
- Store in RT up to 3 months

Hanks buffer

- Composition (for 1 l):
125 mM NaCl (41.7 ml of 3 M stock solution), 5 mM KCl (5 ml of 1 M stock solution), 1.2 mM NaH₂PO₄ (1 ml of 1.2 M stock solution), 1 mM CaCl₂ (1 ml of 1 M stock solution), 1.2 mM MgCl₂ (1.2 ml of 1 M stock solution), 1 μM ZnCl₂ (1 ml of 1 mM stock solution), 10 mM Glucose (1.9817 g), 25 mM HEPES (25 ml of 1 M stock solution), 0,25% BSA (2.5 g for 1 l), pH 7.4 (adjusted with NaOH)
- Store at 4°C up to 3 months

Blocking buffer (make 100-500 ml)

- Hanks buffer, 2% BSA, 0.1% Triton X-100
- Store at 4°C up to 3 months