Tampereen ammattikorkeakoulu

Measurements of Canine Thyrotropin by Means of Cathodic Electrochemiluminescence

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ABSTRACT

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Measurements of Canine Thyrotropin by Means of Cathodic Electrochemiluminescence

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This thesis was made in the Research & Development department of Labmaster Ltd. The objective of this thesis was to explore the possibility of developing an assay capable of measuring canine thyrotropin concentrations on the current Labmaster LUCIA™ platform. The purpose of this thesis was to conduct a feasibility study on new biomarker and to determine the parameters for the assay.

A series of experiments were conducted to test the ability of Labmaster's LUCIA™ analyzer and its cathodic electrochemiluminescence method to measure standard solutions diluted from recombinant cTSH (canine thyroid-stimulating hormone, also known as thyrotropin). The method and parameters were optimized to improve the sensitivity of the assay.

The feasibility study was successful, the measurement results indicate that it is possible to measure cTSH with the current Labmaster LUCIA™ platform and the antibody pair effectively identifies recombinant cTSH molecules. Each parameter change improved the sensitivity of the assay and at best the sensitivity reached a cTSH concentration of 1,0 ng/ml.

Further studies are required to test the functionality of the assay with canine whole blood samples and to determine the dilution factor for whole blood samples. As the result, the required sensitivity of the assay can be estimated, and an attempt can be made to improve the sensitivity to a suitable level.

Should the assay be considered for commercialization in the future, its sensitivity must be improved without compromising its point-of-care measurability. Healthy dogs have exceptionally low levels of TSH, and when factoring in the dilution of whole blood samples, the assay may not be sensitive enough to be used to rule out hypothyroidism. However, if the antibody pair effectively identifies native cTSH molecules, and when an appropriate dilution factor is established, the assay could be used to confirm hypothyroidism.

Key words: cathodic electrochemiluminescence, thyrotropin, thyroid stimulating hormone

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ABBREVIATIONS

CECL	Cathodic electrochemiluminescence
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate
cTSH	Canine thyroid-stimulating hormone
DTPA	Diethylenetriaminepentaacetic acid
ITC	1-(p-isothiocyanatobenzyl)
PBS	Phosphate buffered saline
SiO_2	Silicon dioxide
Tb	Terbium
UP	Ultra-Pure

1 INTRODUCTION

This thesis was made at the Research & Development department of Labmaster Ltd., which manufactures and develops *In vitro* and veterinary diagnostic test kits. The tests are measured at the point-of-care with their developed LUCIA™ Analyzer and patented cathodic electrochemiluminescence technology (Labmaster 2023).

Labmaster Ltd. has developed *In vitro* diagnostics assays on human MxA (Myxovirus resistance protein A) and CRP (C-reactive protein). Veterinary diagnostics assays have been developed for canine CRP, foal IgG (Immunoglobulin G), feline and equine SAA (Serum amyloid A). (Labmaster 2023.)

The objective of this thesis is to explore the possibility of developing an assay capable of measuring canine thyrotropin (aka Canine Thyroid Stimulating Hormone; TSH) concentrations on the current Labmaster LUCIA™ platform. The purpose of this thesis is to conduct a feasibility study on new biomarker and to determine the parameters for the assay, for example dilution buffer, optimal incubation time antibody pair and their concentrations.

Veterinary diagnostics play a crucial role in securing animal health by identifying potential health issues before they become apparent, facilitating quicker diagnosis, and enabling prompt treatment planning. Laboratory testing guarantees precise and high-quality outcomes, while point-of-care diagnostics enable prompt decision-making, easing the concerns of animal owners. These assessments form a fundamental aspect of veterinary care. (Health for Animals n.d.)

Point-of-care testing involves clinical laboratory testing performed near the patient care location, enabling swift generation of test results. This enables the quick implementation of the right treatment, resulting in enhanced clinical or economic outcomes compared to conventional laboratory testing. (Larkins & Thombare 2023.)

2 LABMASTER LUCIA™ ANALYZER

The Labmaster LUCIA™ Analyzer (Picture 1) is a blood test analyzer designed for point-of-care use. The analysis can take place at the point of care, including clinics, veterinary hospitals, stables, and facilities dedicated to animal care. It does not need to be maintained or calibrated by users. (Labmaster LUCIA™ Veterinary Diagnostics 2023.)

PICTURE 1. Labmaster LUCIA™ Analyzer (Picture: Netta Saarinen).

The Labmaster LUCIA™ Analyzer is designed for various test kits, enabling the quantification of specific analytes in blood. Utilizing patented cathodic electrochemiluminescence technology, the Labmaster LUCIA™ Analyzer delivers results within six minutes from a whole blood sample. (Labmaster LUCIA™ Veterinary Diagnostics 2023.)

Test kits manufactured by Labmaster Ltd. contain all the components needed for the analysis, that is test cassettes, dilution tubes for whole blood sample, one NFC (near-field communication) card, instructions for use and quick guide. NFC card has batch and analyte-specific parameters for delivering the correct result. (Labmaster 2023.)

The parts of the test cassette are described in figure 1. The test cassette consists of a piston part and an ampoule part. Piston part has the electric connections and ampoule is filled with washing/measuring buffer. In the middle of the cassette there is a sample application hole, under which there is a membrane, which contains labelled antibodies and a silicon chip coated with the capture antibody.

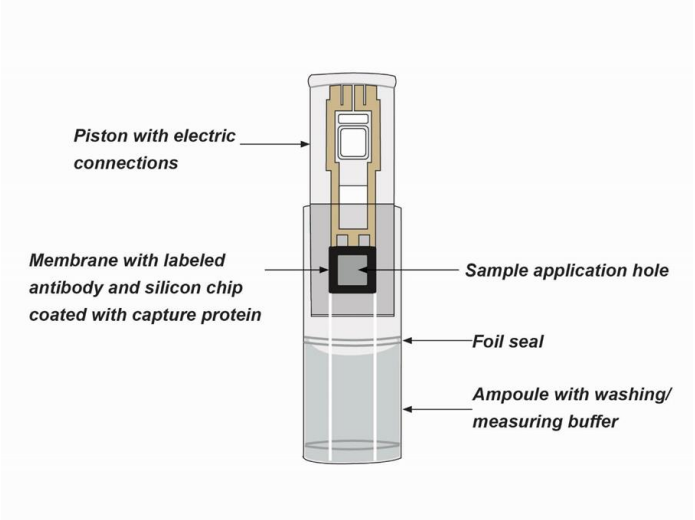
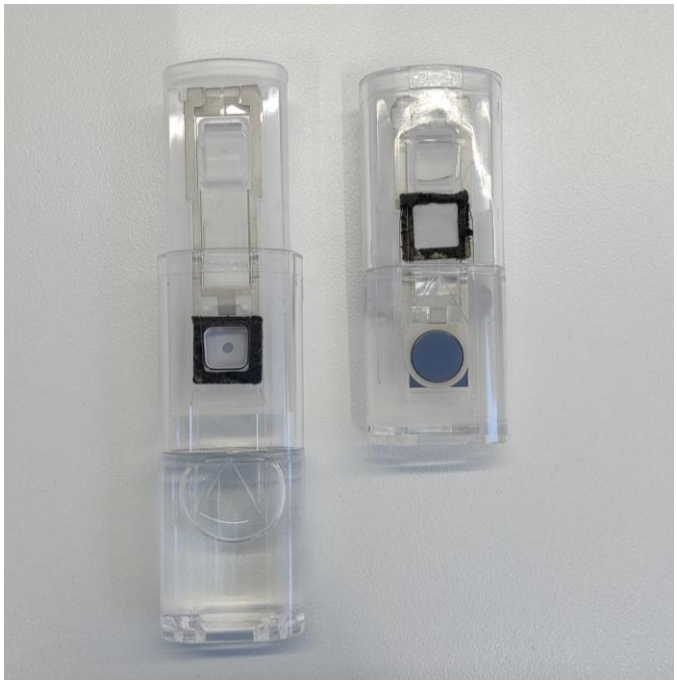


FIGURE 1. Labmaster test cassette (Labmaster 2022).

The difference between an unused and a used cassette is shown in Picture 2.



PICTURE 2. Unused and used cassette (Picture: Netta Saarinen).

3 CATHODIC ELECTROCHEMILUMINESCENCE

Luminescence is an emission of light that occurs when a molecule in an excited state releases energy radiatively to return to its ground state. The various forms of luminescence are distinguished by the source of energy that induces the excited state. Chemiluminescence involves excitation energy provided by a chemical reaction. Electrochemiluminescence, also known as electrogenerated chemiluminescence, is a luminescent process wherein at least one of the reactants leading to luminescence is electrically generated at an electrode, following a straightforward pathway. (Jiang 2006.)

The technology used at Labmaster Ltd. involves the stimulation of a label substance or substances through the electrical excitation by energetic electrons (figure 2). Reactions take place on silicon-based electrodes, which are coated with an insulating layer. (Cathodic electrochemiluminescence LM-CECL 2023.)

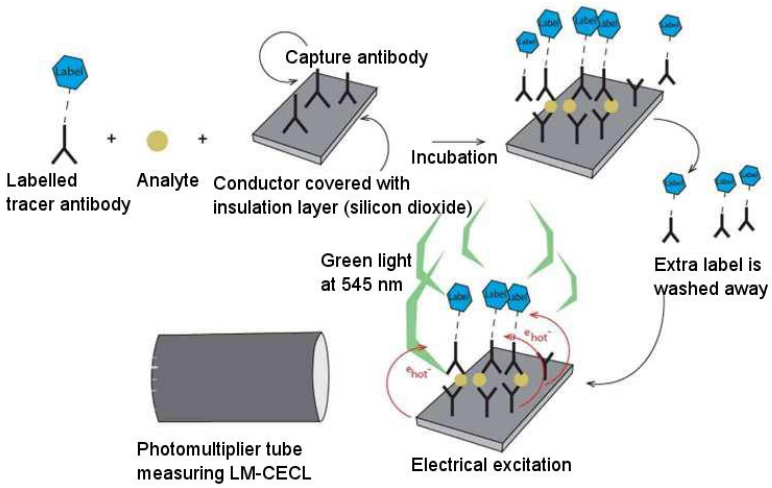


FIGURE 2. LM-CECL (Labmaster Cathodic electrochemiluminescence) (Labmaster 2023).

SiO_2 (silicon dioxide) exhibits various properties relevant to these applications, and depending on the intended function of the oxide, several manufacturing techniques can be employed to produce a SiO_2 layer with the desired properties. One of the techniques is thermal oxidation, where the object is typically oxidized at

temperatures above 1,000°C and can be performed as either a 'dry' or 'wet' oxidation process. (Helin et al. 2001; MKS Instruments 2024.)

In Labmaster devices employing cathodic electrochemiluminescence, aromatic Tb(III) chelate label substance demonstrate robust electrogenerated chemiluminescence when subjected to cathodic pulse polarization on an oxide-covered electrode in an aqueous solution. The method relies on the tunnel emission of hot electrons into an aqueous electrolyte solution. (Cathodic electrochemiluminescence LM-CECL 2023.)

Hot electrons are electrons with energy levels exceeding the Fermi energy of the specific phase. Alternatively, they can be electrons with thermal energies higher than those characteristic of the phase under consideration. (Ylinen-Hinkka 2012.)

The hot electron-induced electrochemiluminescence measurement initiates with cathodic pulse polarization of tunnel junction electrodes consisting of conductor/insulator/electrolyte. These electrodes can transport electrons with significantly higher energy levels into an aqueous electrolyte compared to active metal electrodes or conventional semiconductor electrodes. Throughout the pulse polarization process, hot electrons are emitted through the insulator film via tunnelling. (Ylinen-Hinkka 2012.)

Electrochemiluminescence of aromatic Tb(III) chelates on thin insulating film-coated electrodes provides a highly sensitive method for detecting Tb(III) chelates. This approach is particularly effective when these chelates are utilized as labels in bioaffinity assays targeting biologically relevant compounds. (Ala-Kleme et al. 2006.)

4 NON-COMPETITIVE IMMUNOASSAY

Immunoassays are bioanalytical techniques where the measurement of the analyte relies on the interaction between the analyte and the corresponding antibody. The significance and broad application of immunoassay methods in pharmaceutical analysis stem from their inherent specificity, ability to handle high throughput, and capacity for high sensitivity when analyzing a diverse array of analytes in biological samples. (Darwish 2006.)

Antibodies are molecules, which are produced in animal body during immune response reactions. Immunoassay is a very selective analysis method because antibody can bind extremely strongly to its own analyte. (Ylinen-Hinkka 2012.)

Five distinct types of immunoglobulins are distinguished by their functions, abbreviated as IgA, IgD, IgE, IgG, and IgM. Among these, IgG is predominantly utilized as an immunoassay reagent. IgG is a glycoprotein comprised of multiple subunits, with an approximate molecular weight of 160 kD. It comprises two identical heavy chains and two identical light chains interconnected by disulfide bonds. The constant section is called the Fc (fragment crystallizable region) region, devoid of analyte-binding capabilities. The remaining two segments are identical analyte-binding fragments, referred to as Fab (fragment antigen binding) regions, each equipped with one binding site (figure 3). (Clarke, Sokoll & Rai 2020, 201.)

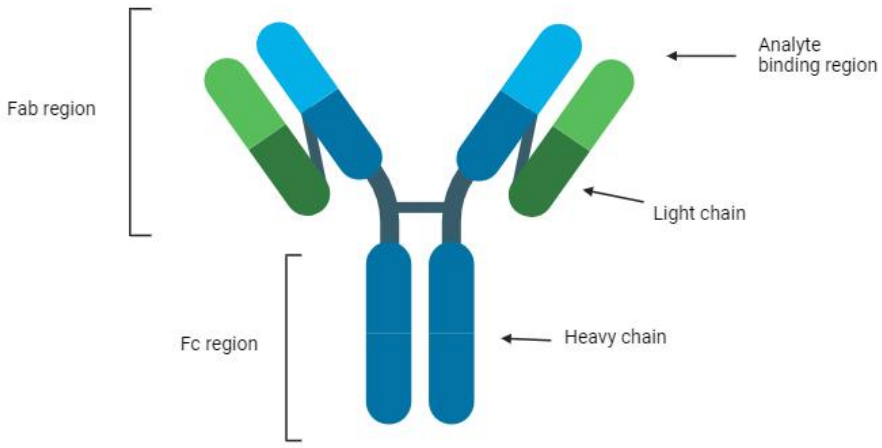


FIGURE 3. Antibody structure (Clarke, Sokoll & Rai 2020, adapted).

The analyte binding site of the antibody is also called the paratope. The interaction between an antibody's paratope and an antigen occurs via non-covalent bonds, encompassing hydrogen bonds, ionic bonds, van der Waals forces, and hydrophobic interactions. (Li et. al 2024.)

The region on an analyte where an antibody attaches is termed an epitope; an analyte may possess numerous epitopes based on its size and molecular composition. Antibodies are categorized as either polyclonal or monoclonal. Polyclonal antibodies encompass a diverse array of antibodies from various B-cell clones, all targeting the same analyte but binding to different epitopes with differing affinities. Monoclonal antibodies, on the other hand, originate from a single B-cell clone and exhibit higher specificity, targeting not just a particular analyte but a precise epitope on that analyte. (Clarke, Sokoll & Rai 2020, 201.)

In non-competitive immunoassays, the signal correlates directly with the quantity of analyte present in the sample. The sandwich immunoassay is the most prevalent non-competitive immunoassay format, employing two antibodies for analyte detection (figure 4). Both polyclonal and monoclonal antibodies are applicable for use in sandwich immunoassays. For this format to be effective, the analyte of interest must be sufficiently large to bind two distinct antibodies simultaneously. The initial antibody in the process, known as the capture antibody, is immobilized to a solid support. It is used to extract the analyte from the sample matrix. A second antibody, labelled as the tracer antibody, interacts with the captured analyte at a separate and unique epitope from the capture antibody, enabling the detection of the analyte once all other components have been removed through washing. (Clarke, Sokoll & Rai 2020, 204.)

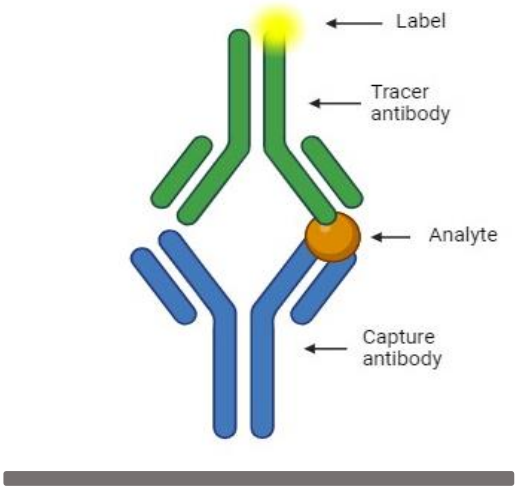


FIGURE 4. Sandwich immunoassay complex (Made with Biorended, Netta Saarinen 2024).

The arrangement of capture proteins, such as antibodies, when immobilized onto a surface is crucial for the effectiveness of immunoassays. The sensitivity of these diagnostic techniques relies heavily on how the antibodies are positioned, ideally with their antigen-binding regions facing the solution. Achieving optimal performance requires the immobilization of antibodies onto a solid surface with the right density, a shape resembling their natural state in solution, and an orientation that enhances their ability to capture antigens. (Welch et al. 2017.)

Physical adsorption stands as the simplest method for this immobilization process, but it lacks control, potentially leading to random orientation, denaturation, or displacement of antibodies during subsequent washing steps (Welch et al. 2017). Physical adsorption of capture antibodies is based on intermolecular forces, mainly hydrophobic, ionic and/or van der Waals interactions (Sarcina et al. 2023).

As antibodies and their binding to analytes do not possess inherently high detectability, employing antibodies as detection reagents requires labelling the antibodies with a detectable reporter molecule. So, in immunoassays, labelled antibodies generate a signal in response to the concentration of the analyte present. Antibodies can be labelled with radioisotopes, fluorophores, luminophores, or enzymes. (Hagan & Zuchner 2011.)

The antibody binding can be detected either directly or indirectly. In the direct method, the label is covalently bonded to the primary antibody. In contrast, with indirect method, the label is covalently linked to a secondary antibody, which then binds to the primary antibody during the immunoassay. (Innova Biosciences n.d.)

Lanthanide can be very useful detectable reporter molecule because it has a remarkably prolonged luminescence. This distinct characteristic enables the selective measurement of lanthanide luminescence even in the presence of other luminescent substances by utilizing time-gating techniques. In essence, the acquisition cycle begins only after the faster decaying background fluorescence has subsided. (Hagan & Zuchner 2011.)

A lanthanide chelator utilized in immunoassays typically consists of two essential components: a chelating group for binding the lanthanide ion and a functional group for attaching to biomolecules. A significant category of these chelators includes polyaminocarboxylates (PACs), such as diethylenetriaminepentaacetic acid (DTPA). (Hagan & Zuchner 2011.)

A chelate is a multidentate ligand that employs multiple atoms to bind to a metal within a coordination complex. In this interaction, the metal serves as the electron-pair acceptor, while the chelating agent functions as the electron-pair donor. Upon binding to the metal ion, the chelate or ligand forms a ring composed of atoms, with the metal being one of the members (figure 5). This chelate complex effectively neutralizes the charge present on the metal ion. (Anderson 2000.)

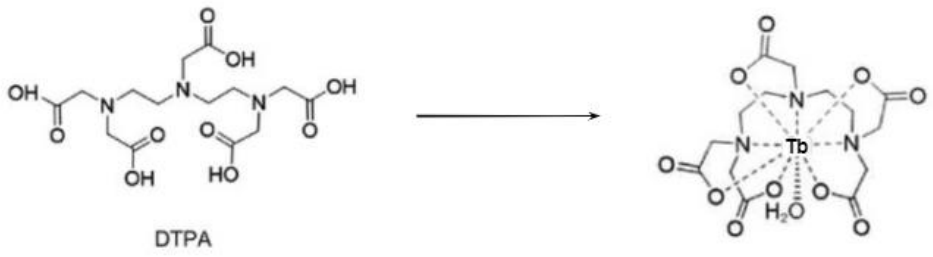


FIGURE 5. DTPA-chelate binding to Terbium (Ding et. al 2018, adapted).

The Tb-DTPA-ITC (Terbium-diethylenetriaminepentaacetic acid-1-[p-isothiocyanatobenzyl]) chelate possesses an aromatic isotiocyanato group, serving as its

reactive component (figure 6). This group undergoes a reaction with primary aliphatic amino groups when exposed to alkaline pH. (PerkinElmer n.d.)

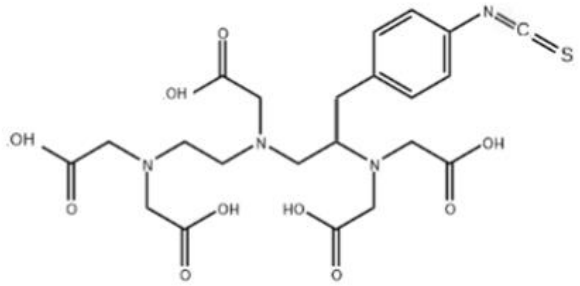


FIGURE 6. DTPA-ITC structure (PubChem 2024, adapted).

The appropriate quantity of Tb chelates attached to an antibody relies on its molecular weight. For antibody with a molecular weight exceeding 100 000, achieving a labelling degree of 4-15 chelates per antibody is considered optimal. Under the specified conditions, when an antibody with a pI of about 6 and a molecular weight of 160 000 is reacted at a concentration of 5 mg/ml, a 40-fold molar excess of chelate over antibody would result in a labelling degree of approximately 8 Tb chelates per antibody. (PerkinElmer n.d.)

5 METHOD DEVELOPMENT AND OPTIMIZATION

The initial stage of assay development involves defining the requirements, encompassing technical limitations, essential materials, and anticipated analytical performance standards. This includes the analyte, the sample matrix, the required detection limit, the measurement range, how specific and accurate the assay should be. Then a suitable analysis format and antibodies are selected. (Wild 2013, 383.)

The selection of antibody pair depends on the target analyte and the accessibility of antibodies recognizing various epitopes on the analyte. Identifying optimal antibodies and correctly assigning their roles requires careful consideration. Typically, the capture antibody exhibits heightened affinity and specificity for the analyte present in the sample, while the tracer antibody might play a lesser role in analyte specificity. (Wild 2013, 387.)

The two important properties of an immunoassay are specificity and sensitivity. In most applications, it is required that the analyte is unambiguously detectable even at low concentrations and even if potentially interfering substances are present in excess. The sensitivity of an assay relies heavily on the achievable signal-to-background ratio. Elevated background signals in blank control samples devoid of analytes can impair the limits of detection and quantification for actual samples, as well as restrict the linear quantification range of an assay. Background interference in luminescence measurements originates from diverse sources, including the sample container or membrane, though typically, the most significant source in biological assays is the sample matrix. (Hagan & Zuchner 2011.)

The sources of error in immunoassays are either exogenous, which are not related to the characteristics of the sample and may reflect system failure, or endogenous, which are usually caused by the sample matrix. Exogenous errors can be random, for example due to variations in sample pipetting or other handling procedures or systematic, for example resulting in consistent deviations from the "true" value due to calibration inaccuracies (Sturgeon & Viljoen 2011.)

On the contrary, endogenous errors typically occur sporadically and are specific to individual specimens, making them challenging to identify. Moderate interference is generally harder to detect compared to severe interference, which is more likely to trigger clinical suspicion based on the results. If left undetected, interference may lead to misinterpretation of results for several reasons. This could include analytical specificity issues where cross-reacting substances resembling the analyte being measured are unintentionally detected, the presence of unusually high concentrations of normal serum, or the formation of antibody or other macromolecular complexes. (Sturgeon & Viljoen 2011.)

In immunoassays where both the tracer and capture antibodies are introduced simultaneously, can be susceptible to the high-dose hook effect when the analyte concentration is extremely high. In this situation, free analytes and bound analytes compete for the binding sites on the solid phase antibodies. Consequently, this competition can lead to erroneously low results, sometimes even falling back within the reference interval. (Sturgeon & Viljoen 2011.)

There are a few challenging issues in protein detection. It can be challenging to detect specific proteins in biological samples due to their highly complex nature and the large number of different proteins, and certain proteins have low expression levels, especially in the early stages of the disease. (Hagan & Zuchner 2011.)

6 THYROTROPIN

Thyrotropin, commonly referred to as TSH (thyroid-stimulating hormone), is a glycoprotein hormone synthesized by the anterior pituitary. Its main function is to serve as the primary stimulus for thyroid hormone synthesis by the thyroid gland. The release of TSH is regulated by the hypothalamic-pituitary axis. Neurons within the hypothalamus release TRH (thyroid-releasing hormone), initiating the stimulation of thyrotrophs in the anterior pituitary to release TSH. Subsequently, TSH induces the release of thyroid hormones, specifically T3 (triiodothyronine) or T4 (thyroxine), from the thyroid follicular cells. (Pirahanchi, Toro & Jialal 2023.)

Thyroid issues can manifest as either hyperthyroidism or hypothyroidism. Hyperthyroidism arises from an excess of thyroid hormone synthesis or release, whereas hypothyroidism results from insufficient production of thyroid hormones (Pirahanchi, Toro & Jialal 2023.) Hypothyroidism, a prevalent endocrine condition in dogs, frequently stems from idiopathic atrophy or immune-mediated thyroid gland destruction (Boretti & Reusch 2004).

In primary hypothyroidism, the thyroid gland fails to produce adequate levels of T3 and T4, resulting in a loss of negative feedback inhibition and increased secretion of TSH from the anterior pituitary gland. When the anterior pituitary gland produces insufficient TSH, there is inadequate stimulation of thyroid follicular cells, leading to decreased levels of T3 and T4, thus resulting in secondary hypothyroidism. (Pirahanchi, Toro & Jialal 2023.)

TSH concentration range in the blood of euthyroid (normal thyroid gland function) dogs is 0,03–0,63 ng/ml (Scott-Moncrieff & Nelson 1998; Boretti & Reusch 2004). According to Movet Laboratoriopalvelut (2022), TSH concentration in the blood of healthy dogs are less than 0,5 ng/ml. TSH concentration range in the blood of hypothyroid dogs is 0,03–19,3 ng/ml (Boretti & Reusch 2004).

Canine TSH can be measured by immunoluminometric assay from a serum or Li-heparin plasma sample (Movet Laboratoriopalvelut 2022). According to Marca et

al. (2001), Canine TSH can be measured with three different methods: immunoradiometric assay, enzyme-immunometric assay and chemiluminescence-immunometric assay.

Thyrotropin can be used in confirming canine hypothyroidism. However, the disease cannot be reliably ruled out due to the limited sensitivity of current cTSH assays. (Boretti & Reusch 2004.)

7 FEASIBILITY STUDY

All test cassettes used in the feasibility study were coated with the capture antibody and labelled with the tracer antibody for this purpose. Used antibodies are referred to as antibody X and antibody Y. Both antibodies were tested as the capture antibody and as the tracer antibody.

7.1 Labelling of the tracer antibody

The molar mass of both antibodies was 155 000 g/mol, so labelling degree of 4–15 was suitable for both. Both antibodies were labelled with Tb-ITC-DTPA chelate. Parameters for the labelling the tracer antibodies are described in Table 1.

Table 1. Parameters for labelling the tracer antibodies.

Parameters	Antibody X	Antibody Y
Amount of antibody	0,53 mg	0,495 mg
pH	10,5	10,5
Buffer concentration	50 mM	50 mM
Temperature	+ 4 °C	+ 4 °C
Reaction time	Overnight	Overnight
Molar excess of Tb-chelate	50x	50x

For the labelling reactions, sodium carbonate buffer was prepared by mixing 1 ml of 500 mM $NaHCO_3$ and 4 ml of 500 mM Na_2CO_3. The pH of the buffer was measured and confirmed to be within the acceptable limits of 10.45–10.54, as the buffer adjusts the pH of the reaction to 10.5.

The mass of the antibody in the solution *m* was calculated using the equation

$$m = c_a \cdot V_a , \quad (1)$$

where c_a is the concentration of the antibody and V_a is the volume of the antibody solution.

The volume of Tb-chelate V_c was calculated using the equation

$$V_c = \frac{50 \cdot \left(\frac{m}{M}\right)}{c}, \quad (2)$$

where 50 is the molar excess of the Tb-chelate, *m* is the mass of the antibody, *M* is the molar mass of the antibody and *c* is the concentration of the Tb-chelate.

The concentration of the sodium carbonate buffer in the reaction must be 50 mM, so the prepared 500 mM sodium carbonate buffer was added to 1/10 of the total volume of the reaction. The pipetted volume of the buffer V_b was calculated using the equation

$$V_b = \frac{(V_a + V_c)}{10}, \quad (3)$$

where V_a is the volume of the antibody and V_c is the volume of the Tb-chelate.

As a calculation example, the labelling of antibody X. 0,5 ml of the antibody X was pipetted into an Eppendorf tube and its mass in the solution was calculated using the equation (1)

$$m = 1{,}06 \frac{\text{mg}}{\text{ml}} \cdot 0{,}5 \text{ ml} = 0{,}53 \text{ mg}.$$

Tb chelate and sodium carbonate buffer were added to the reaction. The Tb-chelate volume was calculated using equation (2) and the buffer volume using equation (3).

$$V_c = \frac{50 \cdot \left(\frac{0{,}00053 \text{ g}}{155\,000 \frac{\text{g}}{\text{mol}}}\right)}{0{,}00474 \frac{\text{mol}}{\text{l}}} = 3{,}607 \cdot 10^{-5} \text{ l} = 36{,}1 \text{ µl}$$

$$V_b = \frac{(36{,}1 \text{ µ} + 500 \text{ µ})}{10} = 53{,}6 \text{ µl}.$$

The antibody Y was labelled using the same equations. The preparation of the label reactions is described in Table 2.

Table 2. Preparation of labelling reactions.

	Antibody X	**Antibody Y**
Volume of Tb-chelate	36,1 µl	33,7 µl
Volume of antibody	500 µl	500 µl
Total volume	536,1 µl	533,7 µl
Buffer volume	53,6 µl	53,4 µl

Both labelling reactions were incubated overnight at a temperature of + 4 °C. After the labelling reactions were sufficiently incubated, the labelled antibodies were purified by exchanging the buffer. First, the label reactions were purified with a Cytiva NAP-5 column, and their eluates were purified with a NAP-10 column. The absorbances of the final eluates at a wavelength of 280 nm were measured using Eppendorf BioPhotometer D30.

Labelling degrees were determined with Victor X4 Multilabel Reader. 10^{-3}–10^{-6} dilutions were prepared from both labelled antibodies. The Victor X4 measured the Tb-signals of the dilutions, blank sample, and the Tb-standard.

The Tb molar concentrations of the dilutions c_D were calculated using the equation

$$c_D = \frac{(s_d \cdot X)}{s_s}, \tag{4}$$

where S_d is the Tb signal of the dilution, X is a correction factor and S_s is the Tb signal of the Tb-standard. The Tb concentrations of 10^{-3}–10^{-6} dilutions were calculated with the same equation.

The initial Tb molar concentration of the labelled antibodies c_{Tb} was calculated using the equation

$$c_{Tb} = \frac{c_D}{DF}, \tag{5}$$

where c_D is the Tb molar concentration of the dilution and DF is a dilution factor.

The concentration of the labelled antibody c_{ab} was calculated based on the measured absorbances at a wavelength of 280 nm using the equation

$$c_{ab} = \frac{(A_{280} - A)}{(\varepsilon \cdot L)} \cdot M, \quad (6)$$

where A_{280} is average of measured absorbances at a wavelength of 280 nm, A is a correction factor, ε is molar extinction coefficient of IgG, L is length of the path and M is molar mass of the antibody.

The molar concentration of the antibody p_{ab} was calculated using the equation

$$p_{ab} = \frac{c_{ab}}{M}, \quad (7)$$

where c_{ab} is the concentration of the labelled antibody and M is the molar mass of the antibody.

The labelling degree LB was calculated using the equation

$$LB = \frac{c_{Tb}}{p_{ab}}, \quad (8)$$

where c_{Tb} is the average of the Tb molar concentrations of the dilutions and p_{ab} is the molar concentration of antibody.

The Tb concentration of the 10^{-6} dilution of antibody X was calculated using the equation (4) and the initial Tb concentration of the labelled antibody X in the 10^{-6} dilution was calculated using the equation (5)

$$c_D = \frac{(5\,964 \cdot 1{,}25)}{429\,746} = 0{,}017347 \text{ nM}$$

$$c_{Tb} = \frac{0{,}017347 \text{ nM}}{0{,}000001} = 17\,347 \text{ nM}.$$

The Tb concentrations of the remaining dilutions were calculated using the same equations. The calculated Tb concentrations of all dilutions were tabulated in Table 3.

Table 3. The calculated Tb concentrations of the labelled antibodies

Dilution	The original Tb concentration of the labelled antibodies (nM)	
	Antibody X	Antibody Y
10^{-3}	14 972	12 348
10^{-4}	15 309	12 877
10^{-5}	16 091	17 644
10^{-6}	17 347	18 412
Average	15 930	15 320

As a calculation example, the concentration of antibody X was calculated using the equation (6), the molar concentration was calculated using the equation (7) and the labelling degree was calculated using the equation (8)

$$\frac{(0{,}0507 - 8\,000\ \frac{\mathrm{cm}^3}{\mathrm{mol\ cm}} \cdot 15{,}9\ \mu\mathrm{m} \cdot 10^{-6} \cdot 0{,}1\ \mathrm{cm})}{(210\,000\ \frac{\mathrm{cm}^3}{\mathrm{M}} \cdot 0{,}1\ \mathrm{cm})} \cdot 155\,000 \frac{\mathrm{g}}{\mathrm{mol}} = 0{,}280 \frac{\mathrm{g}}{\mathrm{l}}$$

$$\frac{0{,}280 \frac{\mathrm{g}}{\mathrm{l}}}{155\,000 \frac{\mathrm{g}}{\mathrm{mol}}} = 0{,}00000181\ \mathrm{mol/l} = 1{,}81\ \mathrm{nmol/ml}$$

$$\frac{15{,}9\ \frac{\mathrm{nmol\ Tb}}{\mathrm{ml}}}{1{,}81\ \frac{\mathrm{nmol\ ab}}{\mathrm{ml}}} = 8{,}8\ \frac{\mathrm{Tb}}{\mathrm{ab}}.$$

The concentration, molar concentration and labelling degree of antibody Y were calculated using the same equations. The calculated concentrations of the antibodies and labelling degrees were tabulated in Table 4.

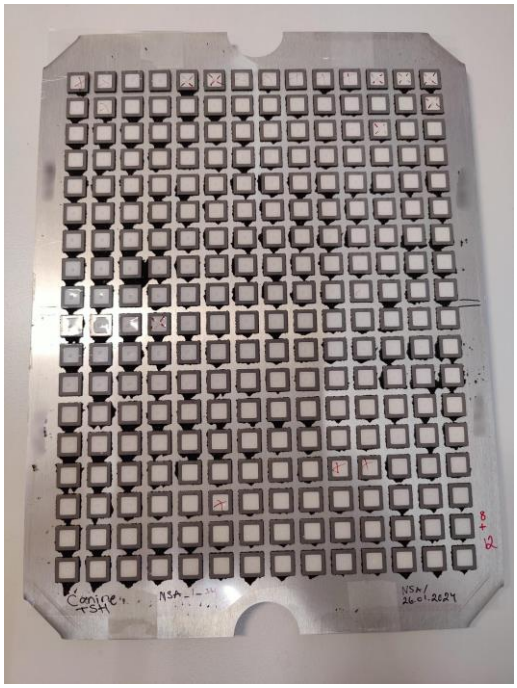
Table 4. Concentrations and labelling degrees of the labelled tracer antibodies.

Antibody	Concentration (mg/ml)	Labelling degree (Tb/ab)
X	0,280	8,8
Y	0,288	8,2

7.2 Labelling of the membranes with the tracer antibody

For each tracer antibody concentration, separate labelling solutions were prepared from both tracer antibodies that were Tb-labelled and purified in an earlier step. Labelling solutions were prepared by diluting the Tb-labelled tracer antibodies with a labelling buffer. The pipetted amounts of the Tb-labelled tracer antibodies and the labelling buffer were calculated using an Excel software template. The labelling buffer and the Tb-labelled tracer antibody were pipetted into an Eppendorf tube. The prepared labelling solutions were vortexed and filtered through a 0,22 µm syringe filter into new Eppendorf tubes.

Membranes were manually labelled with 15, 30 or 50 ng of the tracer antibody per membrane by pipetting 1,0 µl of labelling solution onto the centre of the membranes with a Brand HandyStep Electronic 705000 pipette. If there was a hole in the membrane or a pipetting error occurred, the membrane was rejected and marked with a red marker (Picture 3). One membrane was 8 mm x 8 mm in size.

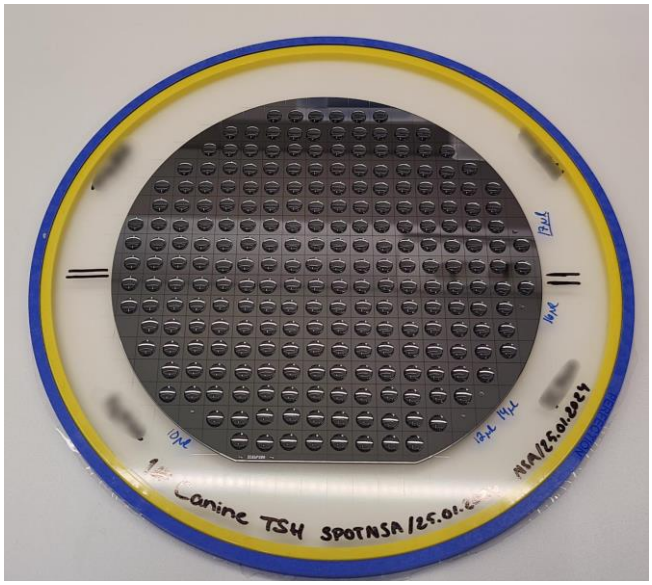


PICTURE 3. Membranes containing the labelled antibody (Picture: Netta Saarinen).

7.3 Coating of the silicon chips with the capture antibody

The SiO_2 chips in the test cassettes were coated manually with coating solutions prepared from unlabelled capture antibodies. Separate coating solutions were prepared for each antibody and concentration.

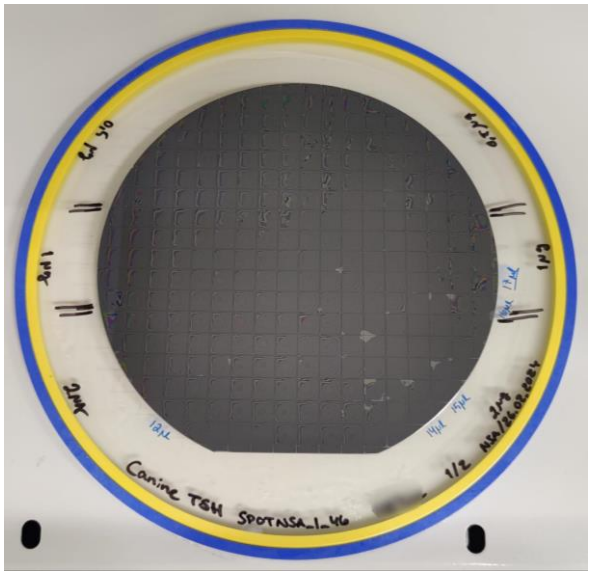
The coating solutions were prepared by pipetting UP water, coating buffer concentrate and the capture antibody into an Eppendorf tube. The pipetted amounts were calculated using an Excel software template. The prepared coating solutions were vortexed and then filtered with a 0,22 µm syringe filter into new Eppendorf tubes. The SiO_2 chips were manually coated with 0.5, 1.0 or 2.0 µg of the capture antibody per chip by dispensing 17 µl of the filtered coating solutions onto the SiO_2 chips (Picture 4) with a Brand HandyStep Electronic 705000 pipette. One SiO_2 chip was 8 mm x 8 mm in size.



PICTURE 4. Wafer of SiO_2 chips coated with the capture antibody (Picture: Netta Saarinen).

The wafers of SiO_2 chips coated with the capture antibody were incubated for 18 hours in a climatic cabinet. The wafers were washed four times with a washing solution. When the washing was completed, the wafers were covered with a saturation buffer and incubated for 2 hours at room temperature. After incubation,

the saturation solution was aspirated with water suction and the wafers were dried in a climatic cabinet for 2 hours until they were dry (Picture 5).



PICTURE 5. Saturated and dried wafer (Picture: Netta Saarinen 2024).

7.4 Experiments

The same LUCIA Analyzer was used for all measurements. Before the first measurement of the day, the functionality of the device was tested by measuring an empty tray and two different light controls.

Standard solutions diluted from recombinant cTSH antigen were used as samples. 0,1 mg of lyophilized cTSH was reconstituted in buffer solution containing 10 mM K-phosphate pH 7.4, 150 mM NaCl, 100 mM D-mannitol and 0.1% CHAPS. 1 ml of buffer was added to the lyophilized antigen. The concentration of the reconstituted antigen was 0,1 mg/ml (100 000 ng/ml).

A working solution was prepared from the reconstituted antigen using equation

$$V_1 = \frac{(c_2 \cdot V_2)}{c_1}, \quad (9)$$

where V_1 is the volume of antigen to be pipetted, c_2 is the concentration of the working solution, V_2 is the volume of working solution to be prepared and c_1 is the concentration of the reconstituted antigen.

$$V_1 = \frac{(1\,000\ \frac{\text{ng}}{\text{ml}} \cdot 1{,}0\ \text{ml})}{100\,000\ \frac{\text{ng}}{\text{ml}}} = 0{,}01\ \text{ml} = 10\ \mu\text{l}$$

Standard solutions were prepared as a serial dilution. The preparation of the solutions is described in Table 5.

Table 5. Preparation of the standard solutions.

Standard solution concentration (ng/ml)	Prepared from (ng/ml)	Volume to be pipetted (µl)	Buffer volume (µl)
1 000	100 000	10	990
100	1 000	100	900
50	100	500	500
10	50	200	800
4,0	10	400	600
1,0	4,0	250	750
0,5	1,0	500	500
0,2	0,5	400	600

7.4.1 Preliminary testing of the antibody pair

The antibody pairs functionality was tested by measuring standard solutions diluted from recombinant cTSH antigen. Antibodies X and Y were both tested as the capture antibody and as the tracer antibody. The concentration of the capture antibody was 2,0 µg, and the concentration of the tracer antibody was 30 ng per cassette.

Labmaster Ltd. normally uses PBS as a dilution buffer, but the cTSH antigen manufacturer recommends diluting the recombinant cTSH antigen dilutions in buffer solution containing 10 mM K-phosphate pH 7.4, 150 mM NaCl, 100 mM D-mannitol and 0.1% CHAPS. Separate sets of standard solutions with cTSH concentrations of 50, 100 and 1 000 ng/ml were prepared into both buffers and two sets of measurements were measured. The buffers were used as blank samples.

The first set of measurements were made using antibody X as the capture antibody and antibody Y as the tracer antibody. The second set of measurements were made using the antibody Y as the capture antibody and the antibody X as the tracer antibody. 10 µl of the standard solution was pipetted onto the cassette and incubated on the table for 5 minutes before measurement.

7.4.2 Assay sensitivity testing

The sensitivity of the assay was tested by measuring lower cTSH concentrations. Standard solutions with cTSH concentrations of 0.2, 0.5, 1.0, 4.0 and 10 ng/ml were diluted into buffer containing 10 mM K-phosphate pH 7.4, 150 mM NaCl, 100 mM D-mannitol and 0.1% CHAPS. The buffer was used as blank sample.

Both antibodies were tested as the capture antibody and as the tracer antibody. The concentration of the capture antibody was 2,0 µg, and the concentration of the tracer antibody was 30 ng. 10 µl of the standard solution was pipetted onto the cassette and incubated on the table for 5 minutes before measurement.

7.4.3 Testing the effect of incubation time

The experiment tested whether a longer incubation time improves the sensitivity of the assay. Cassette incubation time of 10 minutes was tested by measuring standard solutions with cTSH concentrations of 0.2, 0.5, 1.0 and 4.0 ng/m. The buffer was used as blank sample.

Antibody X was tested as the capture antibody and antibody Y as the tracer antibody. The concentration of the capture antibody was 2,0 µg, and the concentration of the tracer antibody was 30 ng.

The standard solutions were diluted into buffer containing 10 mM K-phosphate pH 7.4, 150 mM NaCl, 100 mM D-mannitol and 0.1% CHAPS. To prevent the sample from drying, 20 µl of the standard solution was pipetted onto the cassette and incubated on the table for 10 minutes before measurement.

7.4.4 Testing of different antibody concentrations

In this experiment, the effect of antibody concentrations on the sensitivity of the assay was tested. Capture antibody concentrations of 0,5 and 1,0 µg were tested, and tracer antibody concentrations of 15 and 50 ng were tested. The antibody concentrations were not cross tested, so when the capture antibody concentrations were tested, the tracer antibody concentration remained at 30 ng, and when the tracer antibody concentrations were tested, the capture antibody concentration remained at 2,0 µg. Antibody X was tested as the capture antibody and antibody Y as the tracer antibody.

Standard solutions with cTSH concentrations of 0.2, 0.5, 1.0 and 4.0 ng/ml were diluted into buffer containing 10 mM K-phosphate pH 7.4, 150 mM NaCl, 100 mM D-mannitol and 0.1% CHAPS. The buffer was used as blank sample. 10 µl of the standard solution was pipetted onto the cassette and incubated on the table for 5 minutes before measurement.

7.4.5 Assay testing without membrane material

In the experiment, it was tested whether the sensitivity of the assay can be improved with a membraneless cassette by measuring standard solutions diluted from recombinant cTSH. In this experiment antibody X was tested as the capture antibody and antibody Y as the tracer antibody.

The piston parts of the cassettes were assembled normally, but the labelling was done directly on the SiO_2 chip coated with the capture antibody. The labelling solution was prepared in the encapsulation solution and dispensed 1,0 µl onto the chip. The concentration of the tracer antibody was 30 ng per cassette and the concentration of the capture antibody was 1,0 µg per cassette.

Standard solutions with cTSH concentrations of 0.2, 0.5, 1.0 and 4.0 ng/ml were diluted into buffer containing 10 mM K-phosphate pH 7.4, 150 mM NaCl, 100 mM

D-mannitol and 0.1% CHAPS. The buffer was used as blank sample. 10 µl of the standard solution was pipetted onto the cassette and incubated on the table for 5 minutes before measurement.

7.4.6 Assay testing by preincubating the sample

The experiment tested whether preincubation of the sample with the tracer antibody affects the sensitivity. A solution containing 6,0 µg/ml of the tracer antibody was diluted from the Tb-labelled antibody using equation 9. The solution was prepared into buffer containing 10 mM K-phosphate pH 7.4, 150 mM NaCl, 100 mM D-mannitol and 0.1% CHAPS. Antibody X was tested as the capture antibody and antibody Y as the tracer antibody.

Dilution of antibody Y was calculated using the equation (9), where V_1 is the volume of tracer antibody to be pipetted, c_2 is the concentration of the dilution, V_2 is the volume of dilution to be prepared and c_1 is the concentration of the tracer antibody.

$$\mathrm{V_1} = \frac{(6{,}0\,\frac{\mu g}{ml} \cdot 1\ \mathrm{ml})}{288\,\frac{\mu g}{ml}} = 0{,}0208\ \mathrm{ml} = 20{,}8\ \mu\mathrm{l}.$$

The solution containing 6,0 µg/ml of tracer antibody was mixed in 1:1 with an intermediate dilution of cTSH, resulting a reaction which contains 30 ng/ 10 µl tracer and correct concentration of cTSH. The concentration of the capture antibody was 1,0 µg per cassette.

Two sets of measurements were made. One set of measurements was made when the 1:1 mixture of cTSH and tracer antibody had been pre-incubated at room temperature for 10 minutes and the other set of measurements was made when the mixture had been pre-incubated at room temperature for 1 hour. 10 µl of the mixture was pipetted onto the cassette and incubated on the table for 5 minutes before measurement. The 1:1 mixtures contained cTSH at concentrations of 0, 0.2, 0.5, 1.0 or 4.0 ng/ml.

8 RESULTS

8.1 Preliminary testing of the antibody pair results

The measured emission signals are presented in appendix 1. The emission summary averages, standard deviations, coefficient of variations and signal-to-noise ratio values calculated from the measurement results are presented in Table 6. A graph was created from the concentrations of the standards and the average of the emissions (figure 7).

Table 6. Calculated results for antibody pair testing.

Dilution buffer	Capture + Tracer antibody combination	Sample (ng/ml)	Emission summary average (count)	Standard deviation (count)	Coefficient of variation (%)	Signal-to-noise ratio
PBS	Capture X / Tracer Y	0	688	200	29	-
		50	880	130	15	1,3
		100	1 116	433	39	1,6
		1 000	8 695	1 197	14	13
	Capture Y / Tracer X	0	433	101	23	-
		50	407	39	9,6	0,94
		100	516	118	23	1,2
		1 000	5 359	1 031	19	12
10 mM K-phosphate pH 7.4, 150 mM NaCl, 100 mM D-mannitol, 0.1% CHAPS	Capture X / Tracer Y	0	909	470	52	-
		50	8 527	581	6,8	9,4
		100	12 750	4 818	38	14
		1 000	37 610	7 508	20	41
	Capture Y / Tracer X	0	341	113	33	-
		50	2 991	447	15	8,9
		100	4 340	1 959	45	13
		1 000	18 017	2 892	16	53

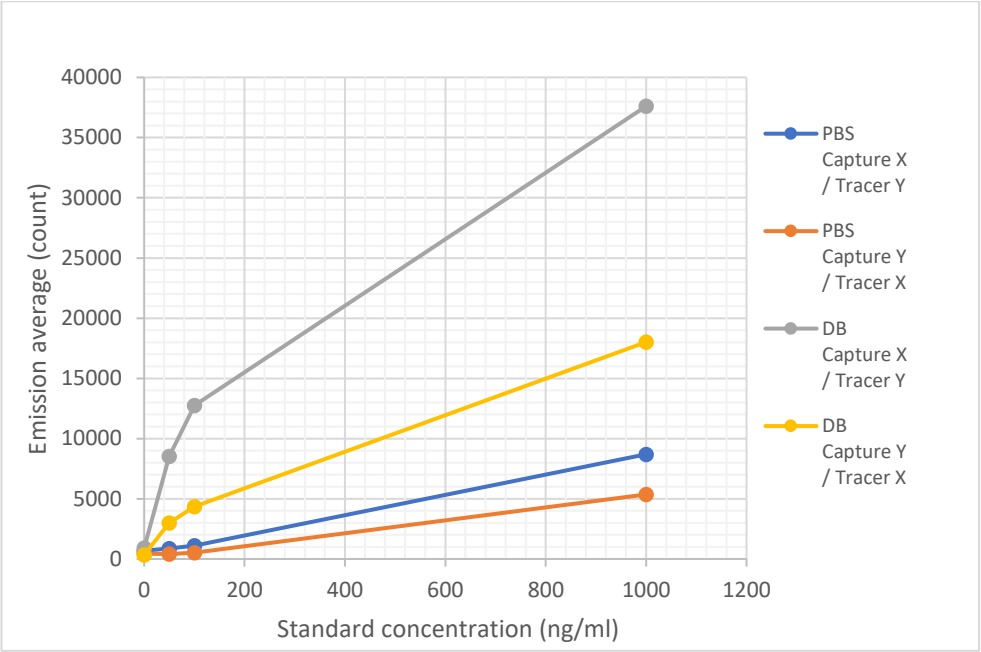


FIGURE 7. Antibody pair testing results

Evidently, PBS was not suitable as a buffer for this analyte. The buffer recommended by the antigen manufacturer (DB) gave significantly higher signals. At these higher concentrations, both antibody pairs recognized the recombinant cTSH molecules, but the antibody pair capture X / tracer Y performed better.

8.2 Assay sensitivity testing results

The measured emission signals are presented in appendix 2. The emission summary averages, standard deviations, coefficient of variations and signal-to-noise ratio values calculated from the measurement results are presented in Table 7. A graph was created from the concentrations of the standards and the average of the emissions (figure 8).

Table 7. Calculated results for assay sensitivity testing.

Capture + Tracer antibody combination	Sample (ng/ml)	Emission summary average (count)	Standard deviation (count)	Coefficient of variation (%)	Signal-to-noise ratio
Capture X / Tracer Y	0	496	80	16	-
	0,2	508	36	7,0	1,0
	0,5	413	66	16	0,83
	1,0	638	76	12	1,3
	4,0	1 400	274	20	2,8
	10	2 268	727	32	4,6
Capture Y / Tracer X	0	300	68	23	-
	0,2	318	44	14	1,1
	0,5	373	102	27	1,2
	1,0	415	90	22	1,4
	4,0	620	99	16	2,1
	10	1 079	193	18	3,6

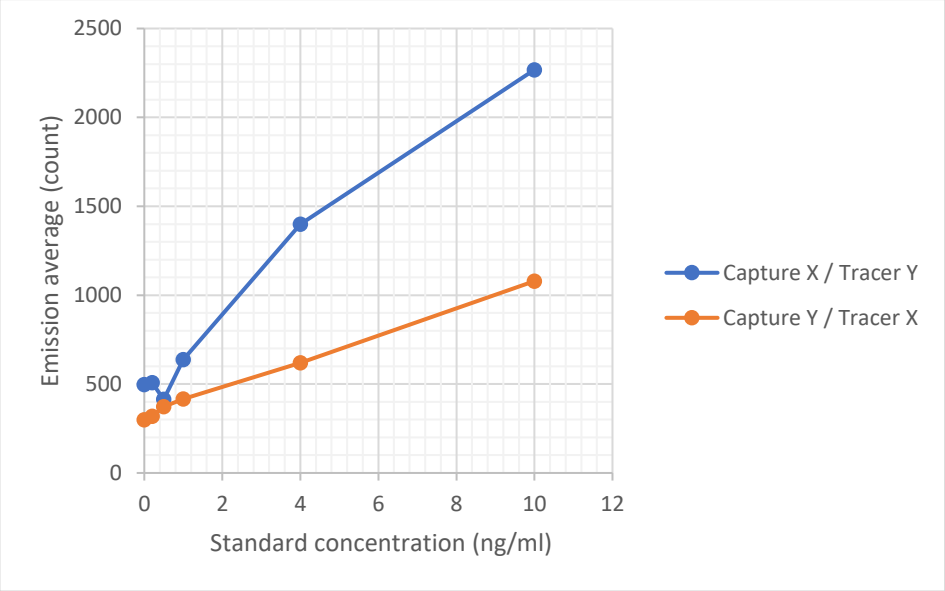


FIGURE 8. Assay sensitivity testing results

The sensitivity of the assay is not sufficient, but it is significantly better with the capture X / tracer Y antibody pair. With this antibody pair and these parameters, the 4,0 ng/ml standard separates from the background signal.

8.3 Testing the effect of incubation time results

The measured emission signals are presented in appendix 3. The emission summary averages, standard deviations, coefficient of variations and signal-to-noise ratio values calculated from the measurement results are presented in Table 8.

A graph was created from the concentrations of the standards and the average of the emissions (figure 9).

Table 8. Calculated results for testing the effect of incubation time.

Capture + Tracer anti-body combi-nation	Incubation time	Sample (ng/ml)	Emission summary average (count)	Standard deviation (count)	Coefficient of variation (%)	Signal-to-noise ratio
Capture X / Tracer Y	10 minutes	0	516	29	5,7	-
		0,2	641	98	15	1,2
		0,5	763	50	6,5	1,5
		1,0	930	63	6,8	1,8
		4,0	2 180	400	18	4,2

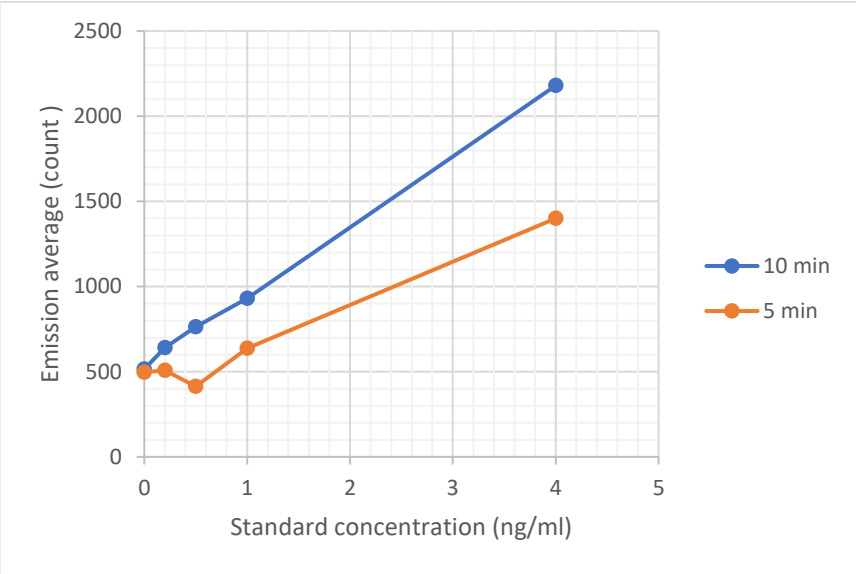


FIGURE 9. Incubation time results

The background signal with a 10-minute incubation time remained in the same level, but the signals of the standards increased, and the linearity improved. Still only 4,0 ng/ml is distinguishable from the background signal.

8.4 Testing of different antibody concentrations results

The measured emission signals are presented in appendix 4. The emission summary averages, standard deviations, coefficient of variations and signal-to-noise ratio values calculated from the measurement results are presented in Table 9 and 10. A graphs were created from the concentrations of the standards and the average of the emissions (figure 10 and 11).

Table 9. Calculated results for testing tracer antibody concentrations.

Capture + Tracer antibody combination	Tracer antibody concentration (ng)	Sample (ng/ml)	Emission summary average (count)	Standard deviation (count)	Coefficient of variation (%)	Signal-to-noise ratio
Capture X / Tracer Y	15	0	216	15	6,7	-
		0,2	209	4,6	2,2	0,99
		0,5	414	120	29	1,9
		1,0	495	90	18	2,3
		4,0	1 347	354	26	6,2
	50	0	969	216	22	-
		0,2	819	131	16	0,85
		0,5	883	64	7,2	0,91
		1,0	1 092	61	5,6	1,1
		4,0	1 770	519	29	1,8

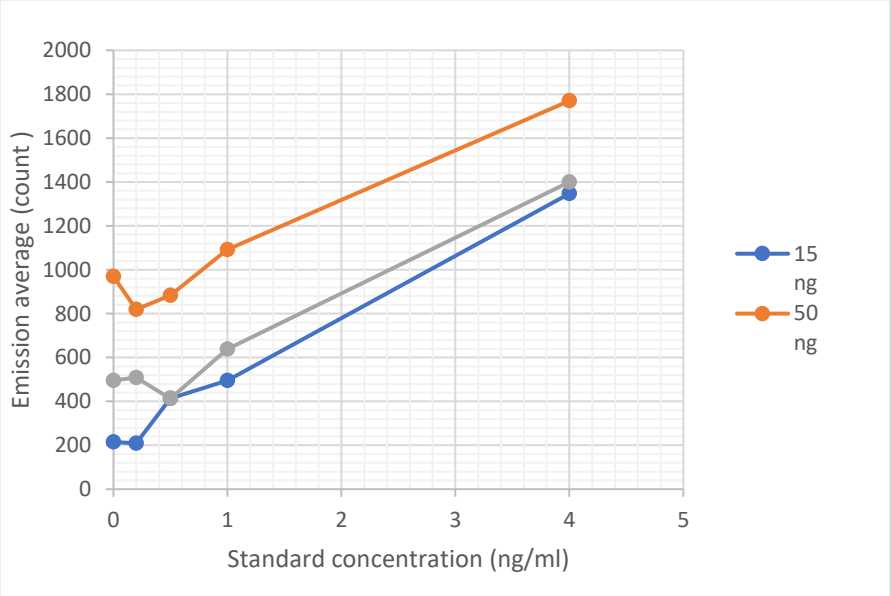


FIGURE 10. Tracer antibody concentration testing

The tracer antibody concentration of 50 ng increases the background signal too much, but the signals of the samples do not increase enough, so the signals of the standards are indistinguishable from the background signal. The 15-ng concentration has the lowest background signal, but the signals are only slightly lower than the 30-ng concentration.

Table 10. Calculated results for testing capture antibody concentrations.

Capture + Tracer antibody combination	Capture antibody concentration (µg)	Sample (ng/ml)	Emission summary average (count)	Standard deviation (count)	Coefficient of variation (%)	Signal-to-noise ratio
Capture X / Tracer Y	0,5	0	404	32	7,8	-
		0,2	433	15	3,5	1,1
		0,5	422	143	34	1,1
		1,0	669	113	17	1,7
		4,0	1 057	131	12	2,6
	1,0	0	308	47	15	-
		0,2	346	58	17	1,0
		0,5	532	218	41	1,5
		1,0	645	68	11	2,1
		4,0	1 419	374	26	4,6

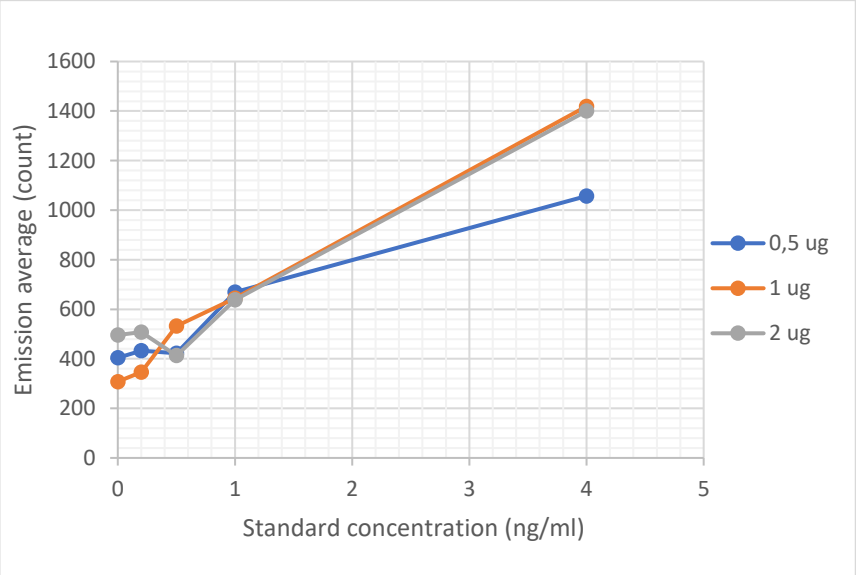


FIGURE 11. Capture antibody concentration testing

At the capture concentration of 0.5 µg, it appears that after a cTSH concentration of 1.0 ng/ml, there could be a hook effect. At the capture concentrations of 1.0 µg and 2.0 µg, the signals are at the same level, but the background signal is lower at the 1.0 µg concentration.

8.5 Assay testing without membrane material results

The measured emission signals are presented in appendix 5. The emission summary averages, standard deviations, coefficient of variations and signal-to-noise ratio values calculated from the measurement results are presented in Table 11.

A graph was created from the concentrations of the standards and the average of the emissions (figure 12).

Table 11. Calculated results for assay testing without membrane material.

Capture + Tracer anti-body combi-nation	Sample (ng/ml)	Emission summary average (count)	Standard deviation (count)	Coefficient of variation (%)	Signal-to-noise ratio
Capture X / Tracer Y	0	486	86	18	-
	0,2	589	39	6,6	1,2
	0,5	760	216	29	1,6
	1,0	928	88	9,5	1,9
	4,0	1 902	69	3,6	3,9

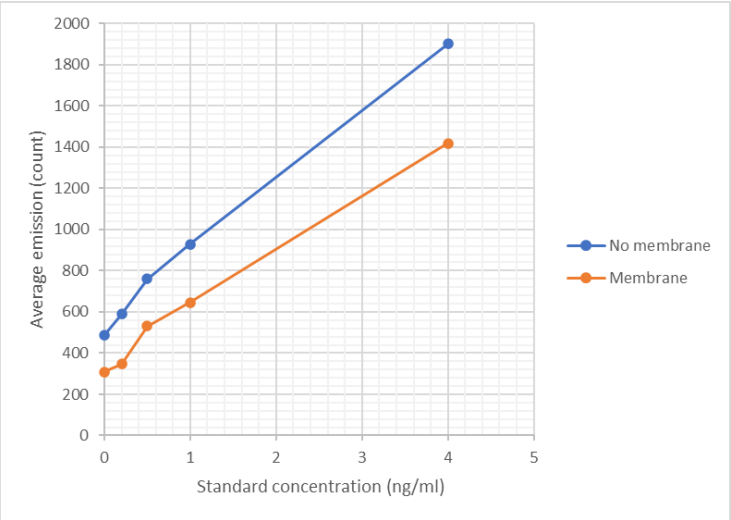


FIGURE 12. Assay testing without a membrane material results

The assay without the membrane slightly increased the background signal, but the signals of the standard solutions also increased. This may indicate a possible binding of recombinant cTSH molecules to the membrane.

8.6 Assay testing by preincubating the sample results

The measured emission signals are presented in appendix 6. The emission summary averages, standard deviations, coefficient of variations and signal-to-noise ratio values calculated from the measurement results are presented in Table 12. A graph was created from the concentrations of the standards and the average of the emissions (figure 13).

Table 12. Calculated results for assay testing by preincubating the sample.

Capture + Tracer anti-body combi-nation	Incubation time	Sample (ng/ml)	Emission summary average (count)	Standard deviation (count)	Coefficient of variation (%)	Signal-to-noise ratio
Capture X / Tracer Y	10 minutes	0	453	68	15	-
		0,2	676	94	14	1
		0,5	802	49	6,1	1,8
		1,0	1 298	141	11	2,9
		4,0	3 859	768	20	9,0
	1 hour	0	418	53	13	-
		0,2	534	88	16	1,3
		0,5	834	128	15	2,0
		1,0	1 067	329	31	2,6
		4,0	2 425	147	6,1	5,8

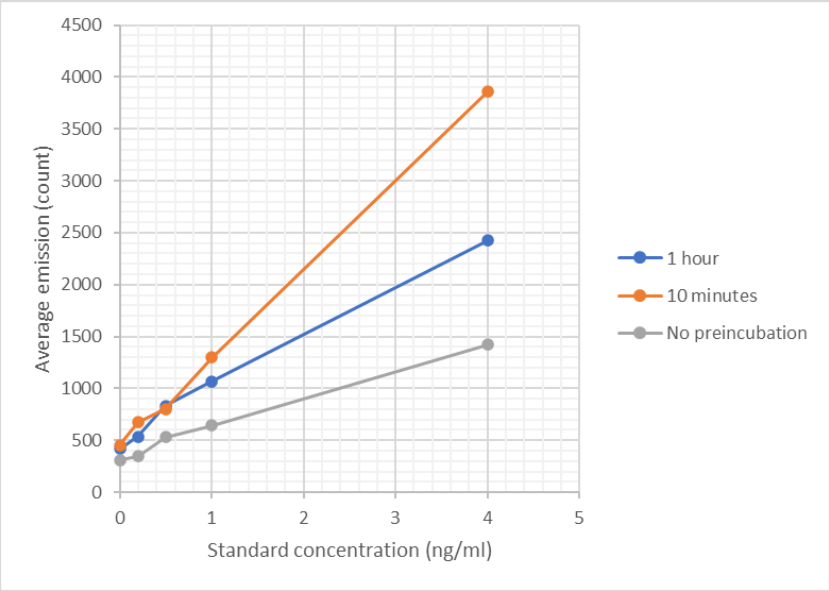


FIGURE 13. Assay testing by preincubating the sample results

The 10-minute preincubation significantly increased the signals of the 1,0 and 4,0 ng/ml standards, but when pre-incubation reached 1 hour the signals started to decrease. This may be because the standards do not last in the buffer at room temperature for long periods of time.

The signals of the 0,2 and 0,5 ng/ml standards increased slightly but are still indistinguishable from the background signal. With a 10-minute incubation, the 1,0 ng/ml standard is separated from the background signal.

8.7 Summary of results

Each parameter change improved the sensitivity of the assay and at best the sensitivity reached a concentration of 1,0 ng/ml. Based on these experiments and their results, the parameters that can be used in further studies were defined.

Antibody X works better as the capture antibody and antibody Y as the tracer antibody. Capture antibody concentrations of 1,0 and 2,0 µg both work, but the background signal is lower with the 1,0-µg concentration, so its use is recommended. A tracer concentration of 30 ng works with both capture antibody concentrations and a tracer concentration of 15 ng works with a capture antibody concentration of 2,0 µg.

The buffer recommended by the manufacturer of the antigen is used as dilution buffer. The sample is preincubated for 10 minutes with the tracer antibody, 10 µl of the sample is pipetted onto membrane-free cassette and incubated for 5 minutes. The parameters are tabulated in Table 13.

Table 13. Assay parameters.

Parameters	
Capture antibody	Antibody X, 1.0 µg
Tracer antibody	Antibody Y, 30 ng
Dilution buffer	10 mM K-phosphate pH 7.4, 150 mM NaCl, 100 mM D-mannitol, 0.1 % CHAPS -buffer
Sample preincubation	10 minutes
Membrane	Without
Sample volume	10 µl
Incubation time on cassette	5 minutes

9 CONCLUSIONS

The objective of this thesis was to explore the possibility of developing an assay capable of measuring canine thyrotropin concentrations on the current Labmaster LUCIA™ platform. The purpose of this thesis was to conduct a feasibility study on new biomarker and to determine the parameters for the assay, for example dilution buffer, optimal incubation time, antibody pair and their concentrations.

In this thesis, a feasibility study was conducted, in which it was verified that the antibody pair recognizes recombinant cTSH molecules. The feasibility study also investigated the sensitivity of the assay and the effect of changes in different parameters. Each parameter changes slightly improved the sensitivity of the assay. Based on the results, it was possible to determine preliminary parameters that can be used in further studies.

The 4,0 ng/ml standard solution was clearly separated from the background signal in every experiment. In the 10-minute pre-incubation of the sample, the concentration of 1,0 ng/ml separated from the background signal, when its signal-to-noise ratio was 2,9. A higher signal-to-noise ratio indicates higher signal over background noise. A signal-to-noise ratio of 3 is commonly considered the lower limit for accurate detection. (Molecular devices n.d.)

The results can be considered reliable because the standard deviation and CV% are mostly within acceptable limits. Opinions vary regarding the appropriate acceptance criteria for CV% to demonstrate parallelism. Some suggest that a CV% of ≤30% for samples in the dilution series suffices, while others advocate for a stricter level of below 20%. (Andreasson 2015.)

The variability in results among parallel measurements is likely attributed to differences in the test cassettes. Efforts are directed towards maximizing the homogeneity of the test cassettes. However, the immobilization of the capture antibody on the SiO_2 chip is not controlled, so their adhesion is not homogeneous. The test cassettes are also assembled by hand, which can cause human error and difference in results.

The objective of the thesis was achieved; the measurement results indicate that it is possible to measure cTSH with the assay and current Labmaster LUCIA™ platform. Further studies are required to test the functionality of the assay with whole blood samples. It is necessary to confirm that the antibody pair can recognize the native cTSH molecules by measuring whole blood samples with high TSH concentration. After that, a suitable dilution factor must be determined for the whole blood sample and verify the compatibility of the dilution buffer by trying different buffers and different dilution factors. The impact of red blood cells in a whole blood sample on the background signal can be examined by measuring samples with TSH concentrations below the measurement range.

The whole blood sample must be diluted because the compounds like immunoglobulins, various proteins, lipids, and bilirubin found in certain blood serum samples have the potential to disrupt certain immunoassays, resulting in inaccurately elevated or reduced results (Sturgeon & Viljoen 2011). Once the appropriate dilution factor has been determined, the required sensitivity of the assay can be estimated, and an attempt can be made to improve the sensitivity to a suitable level.

The sensitivity of the assay can perhaps be improved by experimenting with other parameters or combining changes in different parameters. Either the interaction of the antibodies should be improved or the background signal reduced. One approach to minimize the background signal involves cross testing the capture antibody concentration of 1,0 µg and the tracer antibody concentration of 15 ng. The final testing of the sensitivity can be done with the proof-of-concept method, where the assay is performed in multiple steps. The steps include pipetting a sample onto the coated chip, incubating for 5 minutes, followed by washing the chip, applying the tracer antibody, and incubating for another 5 minutes.

Should the assay be considered for commercialization in the future, it's imperative to enhance its sensitivity without compromising its point-of-care measurability. TSH concentrations in healthy dogs are exceptionally low, and when factoring in the dilution of whole blood samples, the assay cannot be used to rule out hypo-

thyroidism. According to College of Veterinary Medicine (2016), it is recommended to use TSH together with other thyroid tests for diagnosis and the most useful tests for diagnosing hypothyroidism are total T4 and free T4.

Labmaster Ltd. is also developing a total T4 assay for dogs. Therefore, if the antibody pair used in the TSH assay effectively recognizes native cTSH molecules and a suitable dilution factor is determined, the assays can be used collectively to confirm hypothyroidism.

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APPENDICES

Appendix 1. Measurement results of preliminary testing

Dilution buffer	Capture + Tracer antibody combination	Sample (ng/ml)	Emission
PBS	Capture X / Tracer Y	0	870
		0	785
		0	410
		50	876
		50	1 042
		50	723
		100	776
		100	1 727
		100	846
		1 000	10 298
		1 000	7 419
		1 000	8 369
	Capture Y / Tracer X	0	539
		0	297
		0	462
		50	454
		50	358
		50	409
		100	681
		100	455
		100	412
		1 000	5 667
		1 000	6 440
		1 000	3 971
10 mM K-phosphate pH 7.4, 150 mM NaCl, 100 mM D-mannitol, 0.1% CHAPS	Capture X / Tracer Y	0	561
		0	594
		0	1 573
		50	9 027
		50	8 842
		50	7 711
		100	10 502
		100	8 303
		100	19 445
		1 000	48 195
		1 000	31 585
		1 000	33 052
	Capture Y / Tracer X	0	321
		0	488
		0	214
		50	3 622
		50	2 649
		50	2 702
		100	3 760
		100	2 285
		100	6 976
		1 000	21 115
		1 000	14 155
		1 000	18 782

Appendix 2. Measurement results of assay sensitivity

Dilution buffer	Capture + Tracer antibody combination	Sample (ng/ml)	Emission
10 mM K-phosphate pH 7.4, 150 mM NaCl, 100 mM D-mannitol, 0.1% CHAPS	Capture X / Tracer Y	0	566
		0	538
		0	385
		0,2	544
		0,2	520
		0,2	459
		0,5	499
		0,5	340
		0,5	401
		1,0	745
		1,0	589
		1,0	580
		4,0	1 656
		4,0	1 021
		4,0	1 524
		10	2 511
		10	3 011
		10	1 281
	Capture Y / Tracer X	0	389
		0	287
		0	223
		0,2	257
		0,2	354
		0,2	344
		0,5	270
		0,5	511
		0,5	334
		1,0	391
		1,0	319
		1,0	536
		4,0	743
		4,0	501
		4,0	617
		10	814
		10	1 155
		10	1 267

Appendix 3. Measurement results of testing incubation time

Capture + Tracer antibody combination	Incubation time	Sample (ng/ml)	Emission
Capture X / Tracer Y	10 minutes	0	491
		0	499
		0	557
		0,2	523
		0,2	636
		0,2	763
		0,5	737
		0,5	833
		0,5	720
		1,0	923
		1,0	1 011
		1,0	856
		4,0	1 661
		4,0	2 245
		4,0	2 635

Appendix 4. Measurement results of different antibody amounts

Capture + Tracer antibody combination	Tracer antibody concentration (ng)	Capture antibody concentration (µg)	Sample (ng/ml)	Emission
Capture X / Tracer Y	15	2	0	229
			0	224
			0	196
			0,2	215
			0,2	204
			0,2	207
			0,5	293
			0,5	577
			0,5	371
			1,0	454
			1,0	410
			1,0	620
			4,0	1 152
			4,0	1 843
			4,0	1 045
	50		0	1 039
			0	677
			0	1 192
			0,2	849
			0,2	962
			0,2	646
			0,5	957
			0,5	802
			0,5	891
			1,0	1 150
			1,0	1 119
			1,0	1 007
			4,0	1 814
			4,0	2 382
			4,0	1 113
	30	0,5	0	401
			0	367
			0	444
			0,2	454
			0,2	419
			0,2	426
			0,5	585
			0,5	444
			0,5	237
			1,0	579
			1,0	599
			1,0	829
			4,0	1 050
			4,0	900
			4,0	1 221
		1,0	0	369
			0	254
			0	302
			0,2	394
			0,2	264
			0,2	380
			0,5	461
			0,5	308
			0,5	827
			1,0	584
			1,0	611
			1,0	739
			4,0	1 001
			4,0	1 908
			4,0	1 348

Appendix 5. Measurement results of membraneless assay

Capture + Tracer antibody combination	Sample (ng/ml)	Emission
Capture X / Tracer Y	0	606
	0	407
	0	446
	0,2	600
	0,2	631
	0,2	537
	0,5	999
	0,5	475
	0,5	807
	1,0	896
	1,0	840
	1,0	1 049
	4,0	2 000
	4,0	1 861
	4,0	1 846

Appendix 6. Measurement results of sample preincubation

Capture + Tracer antibody combination	Incubation time	Sample (ng/ml)	Emission
Capture X / Tracer Y	10 minutes	0	430
		0	384
		0	546
		0,2	605
		0,2	809
		0,2	615
		0,5	737
		0,5	854
		0,5	1 012
		1,0	1 121
		1,0	1 305
		1,0	1 466
		4,0	3 760
		4,0	2 972
		4,0	4 845
	1 hour	0	387
		0	493
		0	374
		0,2	656
		0,2	493
		0,2	454
		0,5	662
		0,5	874
		0,5	967
		1,0	1526
		1,0	905
		1,0	771
		4,0	2 625
		4,0	2 371
		4,0	2 278